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Importance of a Siderophore in the Pathogenesis and Virulence of Photobacterium Damsela Subsp. Piscicida in Hybrid Striped Bass (Morone Saxatili X Morone Chrysops).

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IMPORTANCE OF A SIDEROPHORE IN THE PATHOGENESIS
AND VIRULENCE OF *PHOTOBACTERIUM DAMSELA* SUBSP. *PISCICIDA*
IN HYBRID STRIPED BASS (*MORONE SAXATILIS* X *MORONE CHRYSOPS*)

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Interdepartmental Program in Veterinary Medical
Sciences through the Department of Veterinary
Microbiology and Parasitology

by

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B.S., Auburn University, 1972
M.S., Auburn University, 1974
May 1996

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DEDICATION

To the memory of my mother, Evelyn Hawke
and to Mae and Earle Cole who taught me to love
and respect the estuarine environment

ACKNOWLEDGMENTS

I wish to extend my gratitude and appreciation to my graduate advisor, Dr. Ronald Thune. His continuous support and encouragement made it possible to remain steadfast in my pursuit of the Ph.D degree until the end. I greatly appreciate all the opportunities he has provided for me. I would also like to thank Dr. Johannes Storz and Dr. David Huxsoll for their support of our fish disease research program. I particularly want to thank Dr. Richard Cooper whose support and ideas continued to motivate me. I am also grateful to the other members of my graduate committee, Dr. David Horohov, Dr. William Todd, Dr. Wayne Taylor and Dr. Richard Goyer for their guidance and helpful suggestions. In the beginning of the project Dr. Joe Newton was very helpful in forming a plan for the pathogenesis studies. More recently, Dr. Al Camus provided valuable suggestions concerning pathological techniques and Mae Lopez cut beautiful sections. Dr. Lisa Stanley-Collins always had helpful comments and suggestions in many areas.

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ABSTRACT

Photobacterium damsela subsp. *piscicida*, formerly *Pasteurella piscicida*, is an important new pathogen of hybrid striped bass cultured in brackish water on Louisiana mariculture farms. Louisiana isolates compared with strains of *P. damsela* subsp. *piscicida* from Greece, Japan, Israel and Chesapeake Bay, USA and were found to be identical in biochemical phenotype and enzymic activity. Small plasmids of 8 and 5 kb were unique to Louisiana strains.

Sequential daily bacteriology and histopathology on hybrid striped bass experimentally infected by immersion revealed that following high doses (13,000 CFU/ml) the gills and spleen are colonized first as early as 24 hours PI, followed by the blood at 48 hours and the liver at 72 hours. Bacteria reached levels as high as 5.7×10^9 CFU/gram in the spleen and 8.6×10^8 in the blood of moribund fish. The spleen contained a significantly higher number of cells than any other organ at all sampling time periods. Histological examination of the gills using semi-thin sections showed high numbers of bacteria invading and colonizing the the gill lamellae at 24 hours PI. Death of the fish appeared to result from respiratory failure due to necrosis of the gill lamellae and blockage of blood flow to the gills by masses of bacteria in the capillaries.

Pathogenic isolates of *P. damsela* subsp. *piscicida* were shown to produce an iron siderophore. A wild type strain 91-197, was mutagenized by introduction of a transposon on pEIS, a suicide delivery plasmid by electroporation. A stable mutant (LSU-P1) deficient in siderophore biosynthesis was generated using this system. LSU-P1 evaluated in an immersion infection model for hybrid striped bass and also by IP injection was found to be non-virulent. The insertion of the transposon into the genome of *P. damsela* subsp. *piscicida* in LSU-P1 was confirmed by PCR analysis using primers to the *kan* gene in the transposon.

INTRODUCTION

The culture of the hybrid striped bass (*Morone saxatilis* x *Morone chrysops*) is a growing industry in the United States and abroad. Production of the hybrid striped bass in the U.S. has increased steadily each year since 1989 and reached 6 million pounds in 1994 (hybrid striped bass growers association, 1995). Although most hybrid striped bass aquaculture is conducted in fresh water systems (Smith et al. 1989), a new mariculture industry utilizing pond, cage, net-pen and raceway culture of this fish has developed in brackishwater sites in coastal Louisiana.

Hybrid striped bass are marketed primarily to white tablecloth restaurants that are willing to pay premium prices for the product. Pond-bank prices for whole fish range from \$2.50 to \$3.00 per pound (Fernandez, personal communication, 1994). Due to strong demand, hybrid striped bass culture is expected to expand substantially and become an an important commercial aquaculture industry within the next 10 years (Hodson et al. 1987).

As in other forms of aquaculture, increased production of hybrid striped bass resulted in an increase in the incidence of disease. Photobacteriosis, or "pasteurellosis", is a devastating disease problem for hybrid striped bass producers in Louisiana. Mortality rates range from less than 5 to greater than 90% in

personal communication 1991; Fernandez, personal communication 1994). The disease has occurred either in the spring or fall, on fish farms in coastal Louisiana every year since December, 1990 (Louisiana Aquatic Animal Diagnostic Laboratory, LAADL, School of Veterinary Medicine, Louisiana State University, 1996). Spring outbreaks of photobacteriosis are characterized by sudden onset of mortality and a very steep mortality curve with 80% mortality occurring in as few as 10 days (Chatry, personal communication 1991). This rapid development with little warning allows an infection to become established in a population before therapy can be initiated. Mortality in fall outbreaks is more prolonged for unknown reasons (Fernandez, personal communication 1995).

Control of photobacteriosis in the U.S. is complicated by the fact that there are no antibiotics cleared by the United States Food and Drug Administration to treat bacterial disease in hybrid striped bass. When emergency permission was granted to use Terramycin® or Romet® medicated feeds, resistant strains of the pathogen emerged (LAADL 1992). The disease is such a devastating problem that many producers failed and the development of a new aquaculture industry in Louisiana is threatened. The remaining producers are investigating the use of vaccines (autogenous whole-cell bacterins, Alpharma Inc., Aquatic Animal Health Division, Bellevue, WA.) to combat the

disease. The vaccines are killed products that are administered by injection or immersion to 40-50 gram fish that are later boosted orally by addition of bacterin to the feed. Producers feel that to be successful a vaccine and an effective antibiotic medicated feed are essential. In the period from December 20, 1990 to December 31, 1995, thirty-two submissions to the LAADL of fish from Louisiana fish farms and one from a fish farm in Israel were diagnosed with photobacteriosis.

The loss of valuable food size fish results, on many occasions, in significant monetary loss. For example, a single production unit houses as many as 44,000 kg of fish valued at \$50,000. Production losses due to photobacteriosis at one Louisiana fish farm in 1991 and 1994 were valued at over 1 million dollars (Fernandez, personal communication 1994). Mariculture of the hybrid striped bass in Israel accounts for production of 200 metric tonnes per year for the European market (Ariav, personal communication, 1994). Photobacteriosis has resulted in severe economic losses in both 1993 and 1994 in Israel.

The causative agent of photobacteriosis is the gram negative bacterium *Photobacterium damsela* subsp. *piscicida*, formerly named *Pasteurella piscicida*. This previously unknown bacterium was initially isolated in 1963 from natural populations of white perch and striped bass during

a massive fish kill in Chesapeake Bay (Snieszko et al., 1964). The authors felt the organism belonged in the genus *Pasteurella* based on physiological and staining characteristics. The bacterium was subsequently studied morphologically, physiologically, and serologically by Janssen and Surgalla (1968) who concluded the organism was a new species and proposed the name *Pasteurella piscicida*. Two additional epizootics, one in Chesapeake Bay (Paperna and Zwerner, 1976) and the other in western Long Island Sound (Robohm, 1979), occurred in wild populations of striped bass. Photobacteriosis was first reported from the Gulf Coast in populations of striped bass cultured in brackishwater earthen ponds in Alabama (Hawke et al. 1987).

Photobacteriosis is also recognized as a serious disease of cultured fish in Japan causing severe economic losses in populations of yellowtail (Kubota et al., 1970). The disease, referred to as "pseudotuberculosis" in Japan, also causes mortality in other cultured fish in Japan, including the red sea bream, the black sea bream, the oval filefish, the red grouper and the striped jack. Recently, photobacteriosis occurred in cultured populations of gilthead seabream in Spain (Toranzo et al., 1991) and Italy (Ceschia et al., 1991), and in sea bass in France (Baudin-Laurencin et al., 1991). Other outbreaks occurred in cultured sea bass in Greece (Maragoudaki, personal communication, 1993) and hybrid striped bass in Israel

(Ariav, personal communication, 1994). Although a variety of fish species are susceptible to *P. damsela* subsp. *piscicida*, the organism has only been isolated from diseased *Morone* spp. in the U.S.

Although several studies report the salient pathological features in fish infected with pasteurellosis (Robohm 1983, Toranzo 1991, Wolke 1975 and Hawke et al. 1987), the pathogenesis of the disease in hybrid striped bass and many other species remains largely unexplored. Prior to studying virulence mechanisms of the bacterium at the molecular level, a description of the pathological sequence of events in the host following exposure to virulent strains of the bacterium is necessary. Waterborne exposure is more representative of the natural route of infection and the pathological events approximate those found in natural infections. After establishing the typical pathogenesis of *P. damsela* subsp. *piscicida*, mutant strains, constructed by transpositional mutagenesis to be negative for siderophore biosynthesis, were examined for their ability to invade, colonize and proliferate in the host. Chapter I summarizes the current literature available on *P. damsela* subsp. *piscicida* with emphasis on virulence mechanisms of the bacterium and of closely related organisms in the genera *Vibrio* and *Photobacterium*. Chapter II provides a phenotypic comparison of strains isolated from Louisiana outbreaks to strains from other

geographic locations. Chapter III describes the sequential pathologic events in hybrid striped bass following immersion challenge with the pathogen. Finally, detection of a siderophore in strain LA91-197, transpositional mutagenesis of the parent strain, and selection of siderophore negative strains are covered in the final chapter. The siderophore is believed to be an important virulence factor in *P. damsela* subsp. *piscicida* (Magarinos et al. 1994). The virulence and pathogenicity of a siderophore negative mutant was assessed using the challenge model developed in Chapter III.

CHAPTER I. LITERATURE REVIEW

Hybrid striped bass

Striped bass spawning and production

The origins of striped bass (*Morone saxatilis*) culture can be traced to Weldon, North Carolina, where, as early as 1884, eggs from ripe wild-caught females were fertilized with sperm from ripe wild-caught males, incubated, and hatched in MacDonald hatching jars. Fry produced using this method were stocked back into the Roanoke River as part of the first conservation program for the striped bass (Whitehurst and Stevens, 1990). Striped bass fingerlings were first produced in 1937 at the Edenton National Fish Hatchery, Edenton, North Carolina, by stocking fry produced at the Weldon hatchery into earthen ponds. The pioneering efforts of Robert Stevens and co-workers in the late sixties led to the development of techniques for hormone induced spawning of striped bass (Stevens, 1966) and the first non-ripe females were hatchery spawned at the Moncks Corner, South Carolina state fish hatchery. The techniques of Stevens, later refined by Jack Bayless, resulted in the production of over 100 million fry per year at the South Carolina hatchery (Bayless, 1972). Improved pond culture techniques followed, resulting in sufficient numbers of

fingerlings to stock most large reservoirs in the southern states and enhance the sport fishery. Techniques for culturing striped bass in brackish water ponds were developed at the Claude Peteet Mariculture Center in Alabama (Powell, 1973; Minton, 1983 and Minton et al. 1985).

Hybrid striped bass production and culture

A hybrid, produced by crossing female striped bass and male white bass (*Morone saxatilis* x *Morone chrysops*), was initially produced in South Carolina for release as a sport fish in natural waters and impoundments (Stevens et al., 1965). This hybrid is referred to as the "original cross" and the approved common name is the palmetto bass. The "reciprocal" cross, produced using female white bass and male striped bass is commonly called the sunshine bass. Hybrids with other members of the genus *Morone* have been produced, but these two remain the most popular for aquaculture (Hodson et al. 1987). For the remainder of this dissertation the term "hybrid striped bass" will refer to the original cross.

The development of commercial aquaculture techniques and culture systems for the production of striped bass, and hybrids thereof, as food fish led to the beginning of a small commercial industry in 1989 (Smith, 1989). The industry experienced tremendous growth in recent years and continues to grow. Hybrid striped bass are presently grown in commercial food fish pond operations in South Carolina,

North Carolina, Virginia, Maryland, Louisiana, Texas, Mississippi, Alabama and Pennsylvania. The feasibility of cage and net-pen culture of advanced fingerlings and subadults was evaluated by Williams et al. 1981 and Woods et al. 1983. Williams concluded that bacterial disease could be a limiting factor in cage culture in marine environments. Highly intensive, semi-recirculating systems utilizing geothermal water and oxygen injection systems are successful in Mississippi and California (Harrell et al. 1990). The development of indoor, closed-loop, recirculating culture systems has offered an alternative form of intensive hybrid striped bass aquaculture (Libey et al. WAS Orlando 1992). Closed systems facilitate aquaculture in climates that are unsuitable for the temperature requirements of the fish or where land is too expensive or groundwater unacceptable for pond culture.

The majority of hybrid striped bass aquaculture is in freshwater systems, however, in coastal Louisiana mariculture of the fish in ponds supplied with saline groundwater or in ponds, net-pens, cages or raceways utilizing natural brackishwater of the coastal marshes is a developing industry.

Fish pasteurellosis

Geographic distribution

Epizootics in the United States

Pasteurellosis of fish, caused by the bacterium *Pasteurella piscicida*, was first documented in the U.S. during the summer of 1963 (Snieszko et al. 1964). Snieszko initially classified the bacterium in the genus *Pasteurella* based on morphological and physical features. Following additional study, the name *Pasteurella piscicida* was proposed by Janssen and Surgalla (1968). Historically, the causative agent of fish pasteurellosis was referred to as *P. piscicida* until it was recently renamed *Photobacterium damsela* susp. *piscicida* and the disease renamed photobacteriosis (Gauthier et al. 1995). It will be referred to as *P. piscicida* in the literature review until the name change occurs.

The original outbreak of pasteurellosis resulted in mass mortality among wild populations of white perch *Morone americanus* and striped bass *Morone saxatilis* in Chesapeake Bay, Maryland. The epizootic started in the Potomac estuary but rapidly spread over a larger area. It was estimated that half the population of white perch (approximately 400,000 kg) were killed in the initial epizootic. Smaller outbreaks were later reported from Long Island Sound in 1977 (Robohm, 1979) and Chesapeake Bay (Paperna and Zwerner, 1976).

Pasteurellosis in a cultured population of striped bass was first reported from juvenile striped bass reared in mariculture ponds for the purpose of tagging and release in coastal waters of Alabama (Hawke et al. 1987). The ponds, located at the Claude Peteet Mariculture Center near the Gulf of Mexico, were supplied with brackish water from the Gulf Intracoastal Waterway. The outbreak resulted in the loss of approximately 80% of the population.

Pasteurellosis caused severe economic losses on Louisiana mariculture farms in populations of cultured hybrid striped bass (*Morone saxatilis* x *Morone chrysops*) from December 1990 until the present (LAADL 1996). Epizootics have occurred seasonally, either in the spring or in the fall when water temperatures are in the range of 17-26°C and salinities are in the range of 3-15 ppt.

Epizootics in Japan

Bacterial infections in cultured yellowtail (*Seriola quinqueradiata*) due to an unidentified bacterium were recorded as early as 1966 in Japan (Kimura and Kitao, 1971). From 1969 to 1972, investigation of heavy mortalities in yellowtail populations cultured in seawater ponds in the southwest of Japan led to identification of the causative agent as *P. piscicida* (Kubota et al. 1970; Kusuda and Yamaoka, 1972; Simidu and Egusa, 1972). In Japan, the disease was termed "pseudotuberculosis" because of the prominent white granuloma-like lesions in the spleen

and kidney of yellowtail. Subsequent studies showed the causative agent of "pseudotuberculosis" to be identical with the Chesapeake Bay isolate of Snieszko (Koike et al. 1975). Fish disease, caused by *P. piscicida*, became common in Japanese mariculture with losses estimated at \$10 million in 1978 in cultured yellowtail and 28% of total disease losses in all species of cultured fish (Egusa, 1980). Currently, photobacteriosis remains a problem in cultured fish (Nakai et al. 1992).

Epizootics in Europe and the Mediterreanean

Prior to 1990 there were no reports of pasteurellosis from Europe. The first account involved juvenile gilthead seabream (*Sparus aurata*) cultured in the northwest of Spain (Toranzo et al. 1991). Epizootics in France in populations of sea bass (*Dicentrarchus labrax*) (Baudin-Laurencin et al. 1991) and in Italy in populations of gilthead seabream (Ceschia et al., 1991) were also reported. Other outbreaks have been described from sea bass in southwestern Spain (Balebona et al. 1992) and Greece (Maragoudaki, personal communication 1993). In 1994, pasteurellosis caused 50% mortality in populations of market size hybrid striped bass cultured in Israel (Ariav, personal communication 1994). Fish and bacterial cultures were shipped to the LAADL for confirmation that the causative bacterium isolated in Israel was *P. piscicida* (Case 94-320). In summary, photobacteriosis (pasteurellosis) is currently a disease of

economic importance in Japan, Spain, Italy, Greece, Israel and the United States.

Erroneous identification

There are four reports in the literature where *P. piscicida* has been erroneously identified as the causative agent of fish mortality. The first involved a fish kill in populations of wild menhaden (*Brevoortia tyrannus*) and striped mullet (*Mugil cephalus*) in Galveston Bay, Texas (Lewis et al., 1970). The organism described in this article is most probably a *Vibrio* based on published biochemical test results. Other reports include; the isolation of pasteurella-like bacteria from rudd (*Scardinius erythrophthalmus*) and chub (*Coregonus zenithicus*) in England (Ajmal and Hobbs, 1967), from brown trout (*Salmo trutta*) and Atlantic salmon (*Salmon salar*) in Norway (Hastein and Bullock, 1976), and from sheat fish (*Silurus glanis*) in Hungary (Farkas and Olah, 1981). The bacteria causing these diseases were later determined to be atypical *Aeromonas salmonicida* (Bullock 1978; Toranzo et al. 1991).

Species susceptibility

A variety of species of fish have been diagnosed with infection by *P. piscicida* in Japan and Europe, but in the United States only fish of the genus *Morone* have been found to be susceptible. The original fish kill on Chesapeake Bay affected populations of white perch (*Morone*

americanus), and to a lesser extent striped bass (*Morone saxatilis*) (Snieszko, et al. 1964). An epizootic in western Long Island Sound was much smaller and involved only striped bass (Robohm, 1979). Other outbreaks in U.S. waters also involved the striped bass (Paperna and Zwerner, 1976; Hawke et al. 1987). In the outbreak at the mariculture facility in Alabama, nine other species of fish present in a reservoir pond where striped bass were experiencing mortality were not affected. During an outbreak on a Louisiana mariculture farm, red drum (*Sciaenops ocellatus*) were unaffected despite being cultured in cages adjacent to those where heavy mortality was occurring in hybrid striped bass (Fernandez personal communication 1994).

In Japan, the cultured species most frequently affected by photobacteriosis is the yellowtail (*Seriola quinqueradiata*) (Kubota et al. 1970). Other affected species of commercial importance in Japan are; the Ayu (*Plecoglossus altevelis*) (Kusuda and Miura, 1972); the black seabream (*Mylio macrocephalus*) (Muroga et al. 1977); the red seabream (*Pagrus major*) (Yasunaga et al. 1983); the oval file fish (*Navodon modestus*) (Yasunaga et al. 1984); the redspotted grouper (*Epinephelus akaara*) (Ueki et al. 1990); the yatabe blenny (*Pictiblennius yatabei*) (Hamaguchi et al., 1991); and the striped jack (*Pseudocaranx dentex*) (Nakai et al., 1992). A case has been reported from the snakehead

(*Channa maculata*), a freshwater fish reared in Taiwan, fed contaminated marine fish products (Tung et al. 1985).

In Europe, species susceptible to photobacteriosis include the gilthead seabream (*Sparus aurata*) (Toranzo et al. 1991; Ceschia et al. 1991) and the sea bass (*Dicentrarchus labrax*) (Balebona et al. 1992; Baudin-Laurencin et al. 1991). Species that have been experimentally infected in laboratory studies include; rainbow trout *Oncorhynchus mykiss*, turbot *Scophthalmus maximus* (Magarinos et al. 1992) and jack mackerel (*Trachurus japonicus*) (Fukui and Kano, 1986).

Taxonomy, morphology and biochemical characteristics

Pasteurella piscicida

Snieszko and colleagues isolated 30 bacterial cultures from moribund white perch and striped bass from the initial epizootic and classified them as being in the genus *Pasteurella* based on morphological and biochemical features (Snieszko et al. 1964). Following additional study, the organism was described as a new species and the name *Pasteurella piscicida* was proposed (Janssen and Surgalla, 1968), although the name was never accepted by bacterial taxonomists and currently lacks taxonomic validity. The following statement is taken from Bergey's Manual of Systematic Bacteriology Volume 1, 1984. "The pasteurella-like fish pathogen described by Janssen and Surgalla (1968) deviates from the above description of *Pasteurellaceae* by

its larger genome, its cryophilia, its lack of nitrate reductase and its unusual host range (Pohl, 1979; Mannheim et al. 1980), nevertheless it does display some DNA base sequence relatedness to several members of *Pasteurellaceae*; this remains to be studied in detail" (Mannheim, 1984). Various investigators made attempts to properly classify the bacterium prior to 1990 but none were successful. The organism was thought to belong in the genus *Corynebacterium* (Kimura and Kitao, 1971) or *Arthrobacter* (Simidu and Egusa, 1972). After studying 12 strains from various locations, Koike et al. (1975) could not support classification in the genus *Pasteurella*. A subsequent study by Pohl (1981), utilizing DNA hybridization techniques, indicated the organism was 30% related to *Pasteurella*, *Actinobacillus* and *Haemophilus*.

More recently, molecular techniques have been employed to classify the organism correctly. Hybridization studies of 16s ribosomal RNA of *P. piscicida* with that of various related genera provided the first evidence that the organism should not be placed in the genus *Pasteurella* (DeLey et al. 1990). This was subsequently confirmed by sequencing the 16S rRNA (Nicolas et al. 1994). In both studies the bacterium appeared to be more closely related to *Vibrionaceae* than *Pasteurellaceae* and, in the latter case, most closely related to the species *Vibrio damsela*. The organism was formally renamed *Photobacterium damsela*

subsp. *piscicida* based on 16s rRNA sequences (Gauthier et al. 1995).

Photobacterium damsela subsp. *piscicida*

Photobacterium damsela subsp. *piscicida* is a gram negative, pleomorphic rod-shaped bacterium, usually 0.5 - 0.8 by 0.7 - 2.6 μm in size, non-flagellated, and usually exhibiting bi-polar staining (Janssen and Surgalla, 1968). Cells in the log phase of growth are typically longer and more rod shaped than those in older cultures which become coccoid or occur in chains. The organism is non-motile in wet mounts (Snieszko et al. 1964) and in glucose motility deeps (Hawke et al. 1987). The bacterium is oxidase and catalase positive and is sensitive to vibriostatic agent O/129 (Robohm, 1983). Optimum growth occurs between 22.5 and 30.0°C, 1.0 to 2.5% NaCl and pH 6.47 to 7.24 (Hashimoto et al. 1985). The halophilic bacterium fails to grow at NaCl concentrations less than 0.5% or greater than 4.0% or at temperatures below 15.0°C or above 32.5°C. Primary isolation is best achieved on BHI agar supplemented with 2% NaCl or on blood agar (2% sheep blood) without added salt, following incubation for 48 hours at 27°C. Individual colonies on blood agar are: greyish-white in color, opaque, 1 mm in diameter, smooth with an entire border, non-hemolytic and clearly visible after 48 hours incubation at 27°C. The colonies are raised and slightly viscid (Snieszko, 1964).

The bacterium is a facultative anaerobe, producing acid but not gas from glucose, mannose, maltose, fructose and galactose (Hawke et al. 1987). In the majority of biochemical tests the organism is non-reactive but gives enough positive results to generate a unique code number (2005004) in the API 20E system (BioM'ereaux-Vitek) (Kent 1982). A phenotypic comparison of strains from diverse hosts and geographic regions revealed that, regardless of the source, all strains exhibited the same biochemical and physiological characteristics (Magarinos et al. 1992). Thus far the only variation among strains is in production of the enzymes α -glucosidase and *N*-acetyl- β -glucosaminidase detected from live cells using the API-ZYM system (BioM'ereaux-Vitek). Italian strains IT-1 and IT-2 and the Japanese strain EPOY 8803-II are positive and negative respectively for the two enzymes, which is the opposite of other strains tested except for ATCC 17911 (Chesapeake Bay, USA) and ATCC 29690 (Japan) which are negative for both reactions (Magarinos et al. 1992). Differences in the production of proteolytic enzymes by the different strains was detectable by radial diffusion using concentrated extracellular products. The Japanese and Italian strains were positive for caseinase and gelatinase by this method, while other strains were negative. A complete profile of biochemical parameters is listed in Table 1.

Table 1. Biochemical and physiological characteristics of *Photobacterium damsela* subsp. *piscicida*.

Characteristic	Reaction ^a
Gram stain	-
Bipolar staining	+
Motility	-
Oxidase	+
Catalase	+
Methyl red	+
Voges-Proskauer	+
Indole production	-
Nitrate reduction	-
Citrate utilization	-
H ₂ S production	-
Oxidation/Fermentation (glucose)	O/F
Gas production from glucose	-
Growth at:	
5°C	-
10°C	-
15°C	+
25°C	+
35°C	-

(table con'd)

Characteristic	Reaction ^a
<hr/>	
Growth in:	
0.0%NaCl	-
0.5%NaCl	+
3.0%NaCl	+
5.0%NaCl	-
Growth on:	
Tryptic soy agar + 2%NaCl	+
BHI agar + 2% NaCl	+
Blood agar (5%sheep blood)	+
TCBS agar	-
MacConkey agar	-
Arginine dihydrolase	+
Lysine decarboxylase	-
Ornithine decarboxylase	-
Tryptophan deaminase	-
β -Galactosidase(ONPG)	-
Urease	-
Gelatinase	V
Caseinase	V
Elastase	-
Lipase(Tween 80)	-
Phospholipase	+
Amylase	-

(table con'd)

Characteristic	Reaction ^a
Hemolysis(sheep erythrocytes)	-
Acid production from:	
Glucose	+
Mannose	+
Galactose	+
Fructose	+
Maltose	(+)
Sucrose	-
Lactose	-
Rhamnose	-
Arabinose	-
Amygdalin	-
Melibiose	-
Mannitol	-
Inositol	-
Sorbitol	-
Glycerol	-
Sensitivity to:	
Vibriostatic agent 0/129	+
Novobiocin	+
Rodent virulence	-

a. Symbols indicate: +, positive; -, negative; (+), weakly positive; V, variable; F, fermentation reaction; O, oxidative reaction.

Structural components important in virulence

Capsule

Conflicting reports are available concerning the presence of a capsule on cells of *Photobacterium damsela* subsp. *piscicida*. Both Snieszko et al. (1964) and Janssen and Surgalla (1968) report the bacterium as being encapsulated. Subsequent descriptions of strains isolated in Japan, Spain, Italy and Greece do not mention the presence of a capsule. A capsule or glycocalyx, if present, could serve as a virulence factor by inhibiting phagocytosis, aid in attachment and/or protect the bacteria from the bactericidal effects of serum complement (Williams 1988; Cross 1990).

Fimbria

Fimbria are proteinaceous hairlike extensions of the bacterial outer membrane that function to aid in bacterial adhesion to eukaryotic cells (Williams 1988). Because of their role in attachment, fimbria have been considered to be virulence factors. Thus far fimbriae have not been reported from *P. damsela* subsp. *piscicida*.

Flagella

Photobacterium damsela subsp. *piscicida* is non-motile and is non-flagellated (Janssen and Surgalla 1968).

Lipopolysaccharides

The envelope of gram-negative bacteria is composed of an inner membrane, a thin layer of peptidoglycan and an

outer membrane. The major constituents of the outer membrane are protein, lipopolysaccharide (LPS) and phospholipids. Bacterial LPS is composed of three elements: Lipid A, a core oligosaccharide (KDO), and o-antigenic side chain composed of repeating units of oligosaccharides. Bacteria with complete LPS o-side chains are referred to as smooth due to the appearance of the colonies on agar plates. The smooth strains are generally more virulent than rough strains that lack the complete o-side chain. The variation in structure and quantity of the o-side chains is mostly responsible for serological differences between bacterial strains (Kabir et al. 1978).

The LPS of *Photobacterium damsela* subsp. *piscicida* has been isolated and subjected to morphological analysis by polyacrylamide gel electrophoresis, gas chromatography, spectrophotometry and immunoblotting techniques (Nomura and Aoki, 1985). The LPS of six *P. damsela* subsp. *piscicida* strains from diverse geographic regions in Japan and one isolate from Chesapeake Bay, USA were found to have identical LPS structures. The purified LPS of strain U-3 (yellowtail, Kochi Prefecture, Japan) was analysed for chemical composition, lethal toxicity to mice and *Limulus* amoebocyte-gelating activity. The LPS, purified by the phenol water method (P-LPS) (Westphal and Jann, 1965) was composed of 0.87% protein, 24.0% sugar and 36.0% fatty acid whereas the LPS purified by the phenol-chloroform-petroleum

ether method (PCP-LPS) (Galanos et al. 1969) was composed of 0.34% protein, 18% sugar and 34.2% fatty acid. The monosaccharide of P-LPS and PCP-LPS was respectively 30.6% and 16.5% hexose; 3.8% and 2.1% heptose; 2.2% and 3.6% pentose; 2.2% and 3.2% 6-deoxyhexose; 3.2% and 2.6% 2-keto-3-deoxyoctonate (KDO); and 2.8% and 2.4% hexosamine. The principal constituents of fatty acids as determined by gas-liquid chromatography (GLC) and confirmed by mass spectroscopy were: lauric acid, 3-hydroxy lauric acid, myristic acid and palmitic acid. The acute toxicity of LPS in mice was LD₅₀ 30.5 mg/kg and 81.6 mg/kg for P-LPS and PCP-LPS respectively. In the *Limulus* amoebocyte assay, *P. damsela* subsp. *piscicida* LPS contained one tenth the activity of *Salmonella minnesota* LPS (Salati et al. 1989).

Resistance to bactericidal effects of serum

Complement mediated lysis of bacteria is one of the major defense mechanisms of the host against invading microorganisms (Doyle and Sonnenfield 1989). The terminal component of the complement cascade is the membrane attack complex (MAC) of complement c9. The transmembrane portion of the MAC forms a structure that creates a pore in the cell wall which causes an osmotic imbalance resulting in death of the bacteria. In most cases the thickness of the lipopolysaccharide layer determines if complement can be deposited on the cell surface in close proximity to the outer membrane. So called "rough" strains that lack long

o-side chains are more susceptible to complement lysis (Finlay and Falkow, 1989). Virulent strains of *Photobacterium damsela* subsp. *piscicida* are resistant to the the bacterial effects of normal rainbow trout (*Oncorhynchus mykiss*) serum while non-virulent strains are sensitive (Magarinos et al. 1994).

Outer membrane proteins

A number of proteins are located in the outer membranes of bacteria, most of them being structural proteins (Doyle and Sonnenfield, 1989). These include the lipoprotein that binds the outer membrane to intramembranous peptidoglycan and a number of porins that function in transport of various size molecules into the cell. Outer membrane proteins may serve as virulence factors by contributing to bacterial attachment and penetration into the host (Hoffmann, 1992). Bacterial surface proteins that provoke phagocytic ingestion of the bacterium by host cells are called invasins or invasion factors but it is not clear how these proteins function in actin rearrangement.

An essential part of high affinity iron uptake systems (siderophores) is the production of outer membrane protein receptors and enzymes involved in the uptake and release of iron from iron chelators (Neilands, 1982). These iron regulated proteins are not normally synthesized in bacteria grown in iron replete media and are detectable only

following growth under low iron conditions (Crosa and Hodges, 1981). Since iron-regulated outer membrane proteins are produced during clinical infection it is possible that the receptors might serve as protective antigens (Griffiths, 1987).

Outer membrane proteins of *P. damsela* subsp. *piscicida* have received very little study. An analysis of total cell envelopes and outer membrane proteins reveals a similar pattern for strains regardless of their geographic origin or fish host. All strains share at least four major outer membrane proteins, with molecular masses of 20, 30, 42 and 53 kDa (Magarinos et al. 1992). Western blot analysis reveals that the OMP of all strains are immunologically related. When various strains of *P. damsela* subsp. *piscicida* are grown in iron restricted media, outer membrane proteins of 105, 118 and 145 kDa are expressed in all strains and a 75 kDa protein that was not correlated to virulence was expressed in some strains (Magarinos et al. 1994).

Non-structural components important in virulence

Plasmids

Plasmids are extrachromosomal, circular, supercoiled DNA molecules that can be stably inherited during bacterial replication. They usually encode non-essential functions but act as accessory sources of DNA that provide unique functions to bacteria such as antibiotic resistance (R-

factors), virulence factors, nitrogen fixation (*Rhizobium*), conjugation (F-factor) and bacteriocins (colicins of *Eschericia coli*) (Freifelder, 1987). Plasmids have been found to confer a number of important attributes to bacterial fish pathogens such as antibiotic resistance (Aoki and Kitao 1985), iron sequestering systems (Crosa et al. 1977), temperature insensitivity (DeGrandis and Stevenson 1982) and resistance to phagocytosis (Stave et al. 1987).

The importance of plasmids in the virulence of *P. damsela* subsp. *piscicida* is currently unknown. The plasmids were examined initially by Toranzo et al. (1983) using four different methods of detection. A combination of the methods of Portnoy et al. 1981, and Kado and Liu, 1981, proved to be the best to characterize different size plasmids. The method of Kado and Liu was more effective in detecting high molecular weight plasmids whereas the method of Portnoy was useful in resolving low molecular weight plasmids. The number of plasmid bands in Japanese strains of *P. damsela* subsp. *piscicida*, as visualized by agarose gel electrophoresis, range from three to seven. Most strains share three plasmid bands with molecular weights of 37, 15 and 5 MDa (Toranzo et al. 1983). American isolates from Chesapeake Bay and Long Island Sound possess two and five plasmid bands, respectively, that are slightly different in molecular weight from the Japanese strains.

Strain ATCC 17911 (Chesapeake Bay) carries plasmids of 20 and 7 MDa molecular weight while strain SB2-KK (Long Island Sound) possesses plasmid bands of 65, 25, 18, 6.5, and 3 MDa.

Plasmid content of representative European, Japanese and American (ATCC 17911) strains reveals similarities in the European and American strains with each containing a 20 and a 7 MDa band (Magarinos et al. 1992). The European strains from sea bass and sea bream also had a 50 MDa band. Japanese strains from yellowtail (MP-7801 and MZS-8001) and red grouper (EPOY 8803-II) possess from two to five bands with only a 37 MDa plasmid being common to all three. Although variability exists in the plasmid profiles of various strains, all of the pathogenic strains isolated in Japanese yellowtail possess two unique plasmids of 5.1 and 3.5 kb in size. These plasmids may function in producing some essential feature affecting the pathogenicity of the organism in fish (Zhao and Aoki 1992). When virulent and avirulent strains from different geographic locations are compared, no individual plasmid or plasmid profile corresponds to a virulent phenotype.

R-plasmids and antibiotic susceptibility

Most strains of *P. damsela* subsp. *piscicida*, regardless of geographic source, have the same antibiotic resistance profile, possessing markers for kanamycin (KM), erythromycin (EM), streptomycin (SM) and sulfadiazine (SD).

Photobacteriosis on Japanese fish farms is complicated by the emergence of drug resistant strains. Drug resistant strains carrying R-plasmids were first observed in 1980 following the heavy use of chemotherapeutics on fish farms (Aoki and Kitao, 1985). Strains resistant to tetracycline (TC) and ampicillin (APC) are now commonly encountered on fish farms in Japan. Two-hundred eighty-one strains collected from diseased yellowtail were subjected to analysis of their resistance patterns and plasmid DNA and transferable R-plasmids were detected in 168 of the strains encoding various combinations of resistance to chloramphenicol (CM), TC, KM, sulphamonomethoxine (SA) and APC (Takashima et al. 1985). In mating experiments with *E. coli* strain RC85 it was found that all the above mentioned drug resistance genes were contained on a single plasmid (Aoki and Kitao, 1985). In a recent study, MIC's were determined for 12 chemotherapeutic agents on strains of *P. damsela* subsp. *piscicida* from *Seriola* reared on Japanese fish farms from 1989 - 1991. Of 175 strains tested, 152 were found to be resistant to combinations of APC, CM, KM, nalidixic acid (NA), SA, TC, and/or trimethoprim (TMP). One hundred and forty nine of the 152 resistant strains were found to carry transferable R-plasmids (Kim and Aoki, 1993).

In 1991 and 1994, strains of *P. damsela* subsp. *piscicida* were isolated from Louisiana fish farms that were

resistant to Terramycin® (oxytetracycline HCL) and Romet® (sulfadimethoxine and ormetoprim). The resistance was due due to acquisition of an uncharacterized R-plasmid (Hawke et al. 1992). Drug resistant strains have not been reported from European outbreaks (Toranzo et al. 1991).

Siderophores

Bacterial growth depends upon the availability of iron. For most pathogenic bacteria, iron is an essential nutrient that is not readily available in the host. Most of the body's iron is found intracellularly, in ferritin, haemosiderin, or heme. Iron found extracellularly in body fluids is bound to the high affinity iron-binding glycoproteins transferrin and lactoferrin. A related protein, ovotransferrin occurs in avian egg white. As a result, although there is an abundance of iron present in body fluids, the amount of free iron is too small to support bacterial growth (Griffiths, 1987). During infection, the host can reduce the amount of iron bound to serum tranferrin. This decrease is called the "hypoferreaemia of infection". Many microorganisms produce siderophores to combat the unavailability of iron in tissues (Lankford, 1973). Siderophores are low molecular weight iron chelators that, along with their receptors, can effectively solubilize iron or remove it from other chelates and transport it into the cell (Neilands, 1982). The best studied "high affinity" systems utilizing

siderophores are those produced by the bacteria of the genera *Salmonella*, *Escherichia*, *Klebsiella*, *Shigella* and *Vibrio*. Most of the knowledge concerning the biosynthesis, transport, regulation, expression, and role in pathogenicity of bacterial siderophores, has been derived from studies conducted on enterobactin and aerobactin in *E. coli* (Neillands, 1990).

Siderophores produced by fish pathogens

Vibrio anguillarum

The best characterized siderophore mediated high affinity iron transport system in a fish pathogen is the anguibactin-mediated plasmid-encoded system of *Vibrio anguillarum* strain 775 (Crosa, 1989). The expression of this system requires a stretch of about 25 kilobases (kb) of the 65-kb plasmid pJM1. Curing *V. anguillarum* strain 775 of the plasmid results in loss of virulence (Crosa et al. 1980), and it has been shown that the portion of the plasmid that plays the major role in the virulence phenotype is the iron uptake region (Actis et al. 1988). This segment has six contiguous, interspersed genes that are involved in biosynthesis of the siderophore as well as components involved in the iron transport process. Also involved is a *trans*-acting factor designated Taf, encoded by a region on the plasmid other than the iron uptake sequences, that functions as a transcriptional activator for the siderophore biosynthetic genes and the iron

transport genes (Crosa 1989, Singer et al. 1991, and Koster et al. 1991). In analyzing different strains of *V. anguillarum* from different geographic locations, the iron uptake system was encoded on pJM1-like plasmids in all cases. Strains isolated from fish on the Atlantic coast had an increased siderophore production phenotype while those from the Pacific coast behaved as the original strain 775. A slight difference in the nucleotide sequence of the *angR* gene was found to account for the difference.

Serotype 01 and 02 are the two most common serotypes of *V. anguillarum* found in disease outbreaks throughout the world. Most members of serotype 01 possess pJM1-like plasmids and carry genes encoding anguibactin. Strains, belonging to serotype 02 possess an enterobactin-like system encoded by chromosomal genes that does not exhibit any homology with the plasmid encoded system (Conchas et al. 1991).

Aeromonas hydrophila

A new siderophore, amonabactin, was described from *A. hydrophila* 495A2 (Barghouthi et al. 1989). Amonabactin is produced by approximately 70% of *Aeromonas* strains tested and is capable of extracting iron from transferrin. It is not known however, if amonabactin is an important virulence factor in *Aeromonas* species. Some strains of *Aeromonas* also produce enterobactin, rather than amonabactin.

Photobacterium damsela subsp. *piscicida*

Strains of *Photobacterium damsela* subsp. *piscicida* isolated from diseased hybrid striped bass cultured in Louisiana were shown to produce a siderophore in our laboratory in 1992. Subsequently, strains of *P. damsela* subsp. *piscicida* from outbreaks in Spain, were shown to produce an uncharacterized siderophore (Magarinos et al. 1994). The role of the siderophore in the pathogenesis of photobacteriosis in any species of fish has yet to be determined. The siderophore of *P. damsela* subsp. *piscicida* is neither a phenolate nor a hydroxymate and it fails to cross-feed siderophore deficient but receptor positive strains of *V. anguillarum* 775::Tn1-5 (pJHC-91) deficient in anguibactin synthesis, *E. coli* LG 1522 deficient in aerobactin synthesis, or *Salmonella typhimurium* *enb-1* and *enb-7*, deficient in enterobactin synthesis (Magarinos 1994). A positive growth promoting effect was seen when supernatants from *P. damsela* subsp. *piscicida*, grown under iron limited conditions, were added to cultures of *P. multocida* grown at levels slightly above the MIC of EDDHA (ethylene-diamine-di(*O*-hydroxyphenyl acetic acid) and visa-versa. This indicates that siderophores produced by *P. damsela* subsp. *piscicida* and *P. multocida* are related. No growth enhancing effect was seen in *P. damsela* subsp. *piscicida* strains administered purified siderophores of other bacteria (aerobactin, enterobactin, anguibactin,

amonabactin, desferal and ferrichrome). The siderophore of *P. damsela* subsp. *piscicida* was also shown to scavenge iron bound to transferrin in an assay where physical contact was not possible (Magarinos et al. 1994).

Alternative mechanisms of iron acquisition

Iron acquisition without siderophores can be accomplished by the direct removal of iron from host iron-binding proteins. This mechanism is highly specific and involves direct contact of transferrin or lactoferrin with cell surface receptors on bacteria. Bacterial pathogens such as *Neisseria gonorrhoea* (Stevenson et al. 1992), *Neisseria meningitidis* (Criado, et al. 1993); (Schryvers and Morris, 1988), *Haemophilus influenzae* type b (Holland et al. 1991), *Pasteurella haemolytica* and *Pasteurella multocida* (Ogunnariwo et al. 1990 and Ogunnariwo et al. 1991) may obtain iron in this way. *P. multocida* produces multocidin, a siderophore-like substance with undetermined function (Hu et al. 1986). Other pathogens such as *Yersinia* spp. (Perry and Brubaker, 1979), *Vibrio cholerae* (Stoebner and Payne, 1988), *Vibrio vulnificus* (Zakaria-Meehan et al. 1988), and *Vibrio damsela* (Fouz et al. 1994) have mechanisms of obtaining iron directly from heme-containing compounds or ferric ammonium citrate.

The fish pathogen *P. damsela* subsp. *piscicida* is able to utilize heme and hemoglobin as a sole iron source and it has been postulated that a membrane receptor for heme

is produced (Magarinos et al. 1994). Pretreating rainbow trout with hemin, hemoglobin or ferric ammonium citrate lowered the LD₅₀ of nonvirulent strains 3 to 4 log units, while little change was observed with previously virulent strains. It was shown that *P. damsela* subsp. *piscicida* obtained iron from transferrin without physical contact, indicating the utilization of a siderophore mediated mechanism.

Toxins and proteolytic enzymes

Many successful pathogens produce extracellular products that afford protection against host defenses, aid in obtaining nutrients for growth and allow for dissemination of the disease agent from the initial site of infection. Fish pathogens of the genus *Aeromonas* are known to produce proteases and hemolysins which are virulence factors (Thune et al. 1982); (Chabot and Thune 1991) and the catfish pathogen *Edwardsiella ictaluri* has been shown to produce chondroitinase (Shotts et al. 1986), which is expressed at higher levels in virulent strains (Stanley et al. 1994). Some fish pathogens, such as *Edwardsiella tarda* (Ullah and Arai, 1983) and *Yersinia ruckeri* (Romalde et al. 1992), are known to produce potent dermatotoxins. The fish pathogen *Aeromonas salmonicida* produces a phospholipase, glycerophospholipid cholesterol acyltransferase (GCAT) that serves as a primary extracellular toxin (Lee and Ellis, 1990). The halophilic pathogen *Photobacterium* (*Vibrio*)

damsela produces a cytolysin (damselysin) with phospholipase D activity (Kreger et al. 1987).

The role of extracellular products (ECP) in the development of pasteurellosis in fish was investigated by Magarinos et al. (1992). They found that *P. damsela* subsp. *piscicida* ECP were strongly toxic for fish (LD₅₀ ranging from 1.0 to 4.6 µg protein per gram fish), fish cell lines, and homoiothermic cell lines. *Photobacterium damsela* subsp. *piscicida* was not found to be pathogenic for mice, however, ECP were toxic for mice within 48 hours following IP injection. The ECP displayed phospholipase activity and were hemolytic for sheep, salmon and turbot erythrocytes, but not trout erythrocytes. The production of proteolytic enzymes differed slightly among strains from different geographic locations. Only the Japanese and Italian strains hydrolyzed casein and gelatin but none displayed elastase activity. Use of the API-ZYM system revealed slight differences in enzymatic activities among the isolates, the first indication of variation in what has heretofore been considered an extremely homogeneous species. Following injection of purified ECP into gilthead seabream, Noya et al. (1995) found an absence of lesions adjacent to the IP injection site and in the spleen. The authors felt the lack of tissue destructive ability was the result of the low proteolytic activity of the ECP. Epithelial and chloride cells of the gills suffered

degenerative changes after the IP injection of ECP, indicating a high sensitivity to the bacterial toxins.

Antigenic homogeneity

The serological homogeneity of ten strains of *P. damsela* subsp. *piscicida* from various sites in Japan and ATCC 17911 (Chesapeake Bay) was originally confirmed by immunodiffusion (Kusuda et al. 1978). The organism was found to possess one heat stable and four heat labile whole cell antigens as well as three heat stable extracellular antigens. The Long Island Sound isolates (Robohm, 1979) and the Gulf Coast isolates (Hawke et al. 1987) were found to cross react with the Chesapeake Bay strain ATCC 17911 by agglutination.

Serological comparisons of European strains from France, Italy and Spain with strains from Japan and the U.S. revealed a high level of antigenic similarity among all strains with cross agglutination titers of 20,480 to 40,960. The serological homogeneity was supported by the fact that all strains possessed the same electrophoretic LPS pattern and outer membrane protein profile. All of the strains shared at least four major outer membrane proteins with molecular masses of 20, 30, 42, and 53 KDa (Magarinos et al. 1992).

Clinical signs

There are acute and chronic forms of photobacteriosis that have been described from various fish species (Thune et al. 1993).

Acute disease

In the acute form, very little gross pathology is observed regardless of the species affected (Bullock 1978, Toranzo 1991, Robohm 1983, Robohm 1979, Hawke et al. 1987). Externally, striped bass appear normal with the exception of pallor of the gills and petechiae in the opercular region, at the base of the fins and inside the mouth. Striped bass also become lethargic and sink in the water column, fail to regulate pigmentation and show an increased rate of respiration. Internally, an enlarged spleen and hemorrhagic kidney provide the only clearly visible gross clinical signs. Other organs and physical features appear normal with the exception of the liver which may be slightly mottled. Yellowtail show evidence of edema and fail to regulate pigmentation. White perch show only slight hemorrhage around the gill covers and the bases of the fins. Diseased gilthead seabream exhibit no apparent external clinical signs except rare individuals that display slight hemorrhagic areas around the head and gills.

Chronic disease

The chronic form of the disease may vary depending on the species affected and whether or not antibiotic feeds

have been administered. In striped bass and white perch, small white miliary lesions may be seen in the swollen spleen and kidney (Bullock 1978; Wolke 1975). In yellowtail chronic lesions are typified by 1 to 2 mm granuloma-like lesions that are composed of masses of the causal bacterium, epithelial cells and fibroblasts. The lesions grossly resemble true granulomas and have resulted in the disease being referred to as "pseudotuberculosis". Chronic lesions in the spleen of gilthead seabream were found only in fish that died after the peak of mortality or when the pasteuellosis outbreak was almost arrested with antibiotic therapy. The lesions seen were very similar to those of the yellowtail with typical whitish pseudotubercles being present in the spleen (Toranzo et al. 1991). The granulomata have been described as a reaction of epithelioid cells when viability of the bacteria is decreased by medication (Kubota et al. 1982 as cited by Toranzo, 1991).

Histopathology

The histopathology of photobacteriosis in naturally infected white perch and striped bass from Chesapeake Bay was first reported by Wolke (1975). He described, in what was obviously a chronic form of the disease, collections of necrotic lymphoid and peripheral blood cells in the spleen, focal areas of hepatocytes undergoing coagulation necrosis

in the liver, and a conspicuous lack of an inflammatory cell response.

In cultured populations of striped bass fingerlings, acute multifocal necrosis of the lymphoid tissue of the spleen, characterized by a loss of cells, coagulation necrosis, karyorrhexis and large colonies of the causal bacteria was common. In the liver, areas of acute multifocal necrosis, with prominent karyorrhexis were common (Hawke et al. 1987). As in the description by Wolke, inflammatory cellular accumulations were absent.

Histopathological changes noted in naturally and experimentally infected gilthead seabream were as follows: The spleen and kidney showed circumscribed, acute necrotic changes and infiltration with blood cells together with masses of bacteria plugging capillaries and interstitial spaces. In the liver there was a moderate multifocal necrosis of the hepatocytes (Toranzo et al. 1991).

In the spleen of experimentally infected yellowtail, bacterial colonies formed 3 days post infection and were surrounded by inflammatory cells. At five days post infection, mature granulomas containing an eosinophilic substance and surrounded by epithelial cells were observed in the spleen. Phagocytes containing bacteria and swollen into large globules were seen in the spleen and kidney (Kumamoto et al. 1986) and it was theorized that swollen

phagocytes blocked capillary blood flow in the organs resulting in ischemia (Kubota 1970).

In an atypical case of pasteurellosis, a pond cultured population of the freshwater snake-head fish *Channa maculata* in Taiwan was infected by feeding contaminated marine fish. The histopathological lesions were very similar to those of the seabream and striped bass with no chronic epithelioid cell granulomata being observed (Tung et al. 1985).

Viability in aquatic environments and transmission

In their original descriptive work, the causative agent of the Chesapeake Bay fish kill was found to survive for only 3 days in brackish water (Janssen and Surgalla 1968). The survivability of the organism in both filtered fresh water and autoclaved Chesapeake Bay water (12 ppt) was studied by Toranzo et al. 1982. The bacterium was found to be very labile in fresh water with viable cells decreasing by four logs within 24 hours and being undetectable after 48 hours. In brackish water the bacterium survived no longer than 4-5 days suggesting that direct fish to fish transmission is necessary for infection. In comparison, *Vibrio anguillarum* was found to remain viable for over 100 days in the same brackish water test system.

Subsequent studies have found that *P. damsela* subsp. *piscicida* may be capable of persisting in the environment

in a viable but non-culturable state in both sea water and sediments (Magarinos et al. 1993). The number of culturable cells in water and sediment were detected from 6 to 12 days post inoculation with numbers typically higher in the sediment than in the water. Using acridine orange counts, non-culturable but viable bacterial cells were detected for a much longer period. When fresh media was added to the test microcosms, the bacteria resumed growth and maintained pathogenicity.

It has been suggested that another species of fish or invertebrate present in the environment where susceptible species are cultured may serve as an asymptomatic carrier and reservoir of infection (Robohm 1983). Thus far a carrier status has not been established for susceptible species (Toranzo et al. 1991) and the mode of transmission and route of infection of this disease is still largely undefined (Thune et al. 1994).

Pathogenicity

In early descriptive accounts of fish pasteurellosis, *Pasteurella piscicida* was thought to be very host specific with only white perch and striped bass being susceptible in the U.S. and yellowtail in Japan. Further evidence of narrow host specificity in U.S. fish populations was contributed by the observation that nine species of marine fish in a reservoir pond failed to develop the disease despite heavy mortalities of striped bass in the same pond

(Hawke et al. 1987). However, with recent attention being given to photobacteriosis following outbreaks in European countries, it is becoming increasingly clear from laboratory pathogenicity studies that the range of susceptible species may be quite broad.

In initial pathogenicity tests done with white perch, the test fish died within 72 hours after injection with 10^7 bacteria (Allen and Pelczar 1967). Subsequently, the LD_{50} was calculated to be 40,000 cells by intraperitoneal (IP) injection in juvenile striped bass (Robohm 1983). In laboratory pathogenicity studies, Magarinos et al. (1992) found *P. piscicida* strains from different geographic regions to be virulent for gilthead seabream *Sparus aurata*, rainbow trout *Oncorhynchus mykiss* and turbot *Scophthalmus maximus*, by IP injection, with LD_{50} values ranging from 10^3 to 10^6 live cells per fish. Interestingly, the bacterium was more virulent in trout and turbot than for the gilthead seabream. *Pasteurella piscicida* was found to be non-pathogenic for mice (Janssen and Surgalla 1968 and Magarinos et al. 1992).

Experimental infections carried out in juvenile striped jack with autogenous strain SJ-9102 by IP injection and immersion "bath" exposure produced LD_{50} values of $10^{3.2}$ cfu/fish and $10^{2.9}$ cfu/ml respectively. In contrast, the LD_{50} for juvenile red sea bream was higher ($10^{6.9}$ cfu/fish) by IP injection but the red sea bream used in the

experiments were larger (Nakai, et al. 1992). Experimental infections with jack mackerel and yellowtail were carried out by IP injection, peroral, and immersion routes by Fukui and Kano (1986). The LD₅₀ value was not calculated but the lowest dose to cause 10-20% mortality in jack mackerel by IP injection was 4×10^5 cfu/fish and by immersion was 1.6×10^5 cfu/ml. No mortalities were produced in jack mackerel by the oral route. In the yellowtail the lowest dose to cause 60-100% mortality by i.p. injection, perorally and by immersion was 4×10^5 cfu/fish, 5.4×10^7 cfu/fish and 1.6×10^5 cfu/ml respectively. Wakabayashi et al. (1977) reported gastral intubation of yellowtail with 9 to 11 mg of cells per 100 grams of body weight resulted in *P. piscicida* levels in the blood within 72 to 120 hours. Tung et al. (1985) conducted two infectivity trials and found the LD₅₀ of *P. piscicida* in snakehead fish to be $10^{1.9}$ cfu per fish by IP injection making it the most susceptible fish on record. Unfortunately the peroral route was not used in these studies. These studies give some insight into species differences in regard to susceptibility and natural route of infection by *P. damsela* subsp. *piscicida* in known hosts and other species from which natural infections have not yet occurred but leave many questions concerning the pathogenesis of pasteurellosis.

Pathogenesis

The mode of transmission, route of infection, mechanisms of avoiding the immune system and many other aspects of the pathogenesis of pasteurellosis have not been definitively established in any species of fish (Thune et al. 1993). A histopathological study of experimental i.p. infections in yellowtail showed that bacteria were phagocytosed by macrophages in several organs, most prominently in the spleen. Bacteria were thought to proliferate within the macrophages and form colonies of different sizes, even though any proof of intracellular replication was lacking (Kubota, et al. 1970). In chronic infections, granuloma formation occurred in the spleen and kidney in areas where bacteria were localized.

Vaccination

Interest in vaccination to protect fish against infection with photobacteriosis began in Japan in the early 1980's as a result of two factors; 1) the emergence of drug resistant strains of the pathogen (Aoki and Kitao, 1985) and 2) the demonstration of protective antibodies in yellowtail recovering from an outbreak of pseudotuberculosis (Fukuda and Kusuda, 1980). Early experiments in yellowtail showed responses to IP injection of formalin-killed *P. piscicida* in Freund's complete adjuvant (FCA). Peak numbers of plaque forming cells occurred in the spleen in 14 days and serum antibody titers

reached 1:640 within 28 days at 17.4°C (Kitao et al. 1981). Yellowtail were found to be protected from *P. piscicida* injection challenge following immunization by IP injection of formalin killed cells in FCA, high pressure spray, mixing antigen with the feed, hyperosmotic infiltration, and immersion. Survival rates following challenge ranged from 60 - 88% in oral and immersion treatments to 100% in injection and spray treatments (Fukuda and Kusuda, 1981). The response of yellowtail to formalin killed (FKB), heat killed (HKB), and live attenuated bacterins (ALB) was examined by Kusuda and Hamaguchi (1988). The FKB and HKB reduced mortality from 81.3% in controls to 57.3% and 78.7% respectively and the ALB reduced mortality to 25.3%. The ALB was also shown to increase phagocytic activity over controls from 4.0% to 19.0%, compared to the FKB and HKB, which produced 4.8% and 8.0% respectively. No difference in antibody titer among the treatments was seen. Increased phagocyte activation and killing of the bacteria was not investigated in this study (Kusuda and Hamaguchi, 1988).

Lipopolysaccharide (LPS) purified from *P. piscicida* and delivered by immersion and spray was more protective (survival 87% compared to 40% in controls) than whole cell (40% survival) or lysed cell preparations (73% survival) when the fish were challenged by gastric intubation of 25 mg of cells per fish (Fukuda and Kusuda, 1985). A vaccine prepared from ribosomal antigens of *P. damsela subsp.*

piscicida and administered by IP injection was compared with vaccines produced from outer membrane fraction (OMF), lipopolysaccharide (LPS), precipitated antigen (PCA) and extracellular products (ECP). Formalin killed cells (FKC) were used as a control (Kusuda et al. 1988). Of all the vaccine preparations tested, ribosomal antigen produced the highest phagocytic activity but produced the lowest amounts of specific antibody against whole cells of *P. damsela subsp. piscicida*.

Even though results of these studies are promising, subsequent research and attempts to produce commercial vaccines using whole cell bacterins have not proven successful. All the attenuated live vaccines tested thus far have used spontaneously attenuated strains that are not suitable for commercial live vaccines because of their propensity to revert back to the virulent form.

Bacterial transformation

Bacterial transformation is a process in which a recipient cell acquires a gene or genes from free DNA molecules in the surrounding medium. Transformation occurs in nature but in the laboratory the process is accomplished by isolating DNA from donor cells and adding the DNA to a suspension of recipient cells (Freifelder, 1987). In most cases, phage or plasmid DNA carrying the genes of interest are transferred to the recipient cells.

Competent cells

Transformation begins with growing the bacterium to be transformed under specific conditions to make it receptive to the uptake of DNA, the uptake of a DNA fragment, plasmid or phage from the surrounding medium and terminates with the recombinational exchange of part of the donor DNA with the homologous segment of the recipient chromosome or transposon insertion. The ability of most bacteria to take up DNA efficiently is limited and even in a species capable of transformation, the DNA can penetrate only a small fraction of the cells in a growing population. The incubation of cells under certain conditions improves the percentage of cells that can take up DNA. A culture of such cells is said to be "competent". Competence is thought to be inducible by certain proteins, referred to as competence factors, which are thought to be generated as a function of growth temperature, growth phase, pH, and degree of aeration (Freifelder, 1987). Competent cells were generated in this study using suggestions outlined for Gram negative bacteria in the Gene Pulser® electroporation manual (Bio-Rad Laboratories, Richmond, California) and for *Escherichia coli* by Dower et al. (1988).

CaCl₂ transformation

Many bacteria can be altered so that they will take up DNA from the surrounding medium for a brief period by treatment with CaCl₂. Bacteria are placed in a solution of

CaCl₂ and subjected to several temperature shifts before the DNA is added. By an unknown mechanism the cell walls become permeable to DNA and the intracellular nucleases are temporarily inactivated (Freifelder, 1987).

Electroporation

Electroporation is a method of introducing foreign DNA into cells by subjecting the cells to electrical shock. It has been used successfully with both eukaryotic and prokaryotic cells. Basically, competent bacterial cells are mixed with DNA containing the gene of interest in a low ionic strength medium and are exposed to a momentary high voltage charge. For a short time following the pulse, small pores open in the cell wall allowing for DNA uptake then the pores reseal (Sowers 1992).

Transpositional mutagenesis

Transposable genetic elements have become important tools in the study of bacterial genetics. Transposons are mobile elements of DNA that relocate at low frequency. Transposons are excised from the genome of a plasmid or chromosome by means of a transposase enzyme that is encoded by a gene located within the transposon itself, and relocate at certain sites in another portion of the plasmid or chromosome. The resulting insertion often causes a frame-shift mutation that disrupts expression of a particular gene. A characteristic of transposable elements

is the ability to insert in many different locations due to insertion sequence (IS) elements or sequences characterized by inverted repeats which flank the transposon at either end. The IS elements recognize so called "target" sequences in the DNA and insert at these sites. Although no transposon inserts in a target site completely at random, the degree of specificity is low enough that insertions in a specific gene of interest can usually be identified (Kleckner et al. 1991). Transposons occur naturally in many different classes of organisms and are used by bacterial geneticists to introduce mutations into target genes in the bacterial chromosome (Kleckner et al. 1991).

With the evolution of transposon induced mutagenesis, many different types of specialized transposon derivatives and delivery vehicles have been constructed. These transposon cassettes are coupled with a particular delivery vehicle to introduce the transposon into a target bacterial strain that is to be mutagenized. The particular transposon/delivery-vehicle combination must be carefully selected to obtain the desired result. Genetically engineered transposons contain a selectable genetic marker such as antibiotic resistance that allows for selection of the bacterial clone into which the transposon has inserted. Transposition of an element from a nonreplicating phage or plasmid into a stable replicon are the most popular

approaches. Because it is desirable for the delivery vehicle not to persist in the host strain once transposition has occurred, special plasmids called "suicide" vectors have been developed. In one type of suicide plasmid, the plasmid DNA is linearized when the transposon is excised by transposase and the linear DNA is degraded by the endonucleases of the host strain. Another type of suicide plasmid (Miller and Mekalanos 1988) utilizes the π protein dependent origin of replication of plasmid R6K which can only be maintained in certain λ pir lysogens of *E. coli* strains that produce the π protein.

The most widely used constructs are derived from (IS)-based elements (Tn10 and Tn5) or from bacteriophage Mu. Minitransposons, transposons which have the transposase gene located outside their boundaries, are utilized because of the stability of inserts. With minitransposons, problems resulting from secondary transposition events are eliminated because the transposase gene is retained on the donor molecule and is lost following transposition and suicide of the donor molecule (Kleckner et al. 1991). The use of a selectable marker, such as antibiotic resistance, in the transposon cassette allows for selection of clones into which the transposon has inserted. This can be combined with a method of screening for expression of the gene of interest to allow for selection of mutants.

Minitransposon Tn10 derivative 103

Tn10 - 103 is a mini-Tn10 derivative developed by Kleckner et al. (1991). The 1.8 kb transposon carries short segments of Tn10 ends in inverted orientation at each terminus. Derivative 103 carries a marker for Kanamycin resistance Kan^R derived from Tn903. The derivative also carries the double mutant transposase gene *ats1 ats2* fused to a strong isopropyl- β -D-thiogalactopyranoside (IPTG)-inducible *Ptac* promoter (*Ptac*-ATS transposase). ATS stands for altered target specificity. The mutant transposase utilizes a much larger number of insertion sites than the wild type.

Plasmid vehicle pNK2859

The plasmid vehicle pNK2859 is a pBR322 based plasmid carrying the marker for Amp^R, but with a deletion of base pairs 75 to 2353 (Kleckner et al. 1991). The *HindIII* site in this plasmid has been destroyed by filling in. The *EcoRI* fragment, carrying mini-Tn10 derivative Tn103 containing the Kan^R marker, is carried on a *EcoRI* fragment which is inserted into the *EcoRI* site in the plasmid vehicle in the appropriate orientation. The *Ptac* promoter is oriented so it is transcribing in the opposite direction of the *bla* gene, encoding ampicillin resistance.

Plasmid vehicle pEIS

The plasmid vehicle pEIS, constructed in the laboratory of Dr. Richard Cooper, is a hybrid between

pNK2859 (Kleckner et al. 1991) and plasmid pGP704 (Miller and Mekalanos 1988). The 4.2 kb *EcoRI* fragment of pNK2859, containing the minitransposon cassette with the Kan^R marker, is ligated into the multicloning site of pGP704. This results in a transposon inducible by IPTG, carried on a 7.9kb suicide plasmid vehicle, which cannot replicate in a host bacterium that fails to produce the π protein (Cooper, personal communication 1995).

There have been no published accounts of transposon mutagenesis of *Photobacterium damsela* subsp. *piscicida*. Crosa et al. (1989) reported using transpositional mutagenesis in the marine fish pathogen *Vibrio anguillarum* with Tn1 and Tn3-HoHo1 to produce siderophore deficient or outer membrane receptor deficient mutants but it was not stated if electroporation, transformation or conjugation was used to introduce the transposon into the marine bacterium. Mutagenesis of *Vibrio anguillarum* was also accomplished using plasmids pSE6-kan1, pKS1-kan2 and pSE30-kan3 maintained in *E. coli* and delivered by conjugation (Singer et al. 1991).

Study objectives

The marine bacterium *Photobacterium damsela* subsp. *piscicida* has recently become a serious impediment to the development of hybrid striped bass aquaculture in the coastal parishes of the state of Louisiana (LAADL 1996).

This represents the first report of this organism causing disease in a commercially cultured fish species in the U.S. Louisiana isolates of the bacterium have not received detailed study comparing them with important pathogenic strains from other geographic locations. The bacterium has been reported to be a homogeneous taxon in respect to biochemical phenotype, antigenicity, enzyme activity and certain structural components. An initial objective was to compare isolates from Louisiana with strains from other host species from the U.S., Japan, Europe and the Mediterreanean.

Although salient gross and histopathological changes have been published for white perch, striped bass, yellowtail and sea bream, little is known concerning the pathogenesis of photobacteriosis in hybrid striped bass or any other species of fish. One of the original goals of this study was to examine the progress of infection following various routes of administration and to develop a model for photobacteriosis in hybrid striped bass.

An important virulence mechanism in other marine fish pathogens is the production of low molecular weight high-affinity iron binding molecules (siderophore). The discovery in our laboratory that *P. damsela* subsp. *piscicida* produces a siderophore led to the hypothesis that the molecule was an important virulence factor in the development of photobacteriosis. To test this hypothesis,

techniques necessary for the production of siderophore biosynthesis mutants were developed. The additional goal was to test the mutant(s) produced in the infection model for hybrid striped bass.

Specific objectives were: 1) To compare Louisiana isolates of *P. damsela* subsp. *piscicida* with representative strains from the U.S. (Chesapeake Bay), Japan, Greece, and Israel in the areas of biochemical phenotype, enzyme activities, native plasmids, antigenic cross-reactivity, and antimicrobial susceptibility; 2) To establish if juvenile hybrid striped bass can be infected by immersion, IP injection and/or oral intubation; 3) To follow the progress of infection following immersion exposure by quantifying bacterial numbers reaching the internal organs and histological examination of the organs and tissues; 4) To develop a technique for transpositional mutagenesis of *P. damsela* subsp. *piscicida*; 5) To use transpositional mutagenesis to produce a mutant strain of *P. damsela* subsp. *piscicida* deficient in a gene necessary for siderophore biosynthesis; and, 6) To test the virulence of the mutant strain in the model for hybrid striped bass infection.

CHAPTER II. MOLECULAR AND PHENOTYPIC CHARACTERIZATION OF STRAINS OF *PHOTOBACTERIUM DAMSELA* SUBSP. *PISCICIDA* ISOLATED FROM DISEASED HYBRID STRIPED BASS CULTURED IN LOUISIANA

Introduction

The culture of hybrid striped bass (*Morone saxatilis* x *Morone chrysops*) is a growing industry in the United States, with production increasing from 500,000 pounds in 1987 to approximately 6 million pounds in 1994 (Striped Bass Growers Association, 1995). Mariculture farms constructed in the coastal marshes of Louisiana utilizing pond, net-pen, raceway and cage-culture techniques show great promise for the culture of hybrid striped bass; however, since the fall of 1990, yearly outbreaks of photobacteriosis have had a devastating effect on production.

The causative agent of the disease previously known as "fish pasteurellosis" was originally described following a massive fish kill in Chesapeake Bay that destroyed approximately 50% of the natural populations of white perch (*Morone americanus*) and striped bass (*Morone saxatilis*) (Snieszko et al. 1964). Based on a variety of physical and biochemical characteristics the bacterium was tentatively placed in the genus *Pasteurella*. The non-motile gram negative rod was fermentative in glucose, oxidase positive and exhibited bi-polar staining. The pathogen was studied morphologically, physiologically and serologically by

Janssen and Surgalla (1968), who concluded that the bacterium was a new species and proposed the name *Pasteurella piscicida*. The name was never given validity by bacterial taxonomists due to physiological inconsistencies with the genus *Pasteurella*, including lack of nitrate reductase, tolerance to pH values outside the normal range, halophilia, lower optimum growth temperature and unusual host range. The bacterium was not included in Bergey's Manual of Systematic Bacteriology (Mannheim 1984) or the "Approved List of Bacterial Names" (Skerman et al. 1989). Nevertheless, the name was used in the literature until 1995 when the organism was formally renamed *Photobacterium damsela* subsp. *piscicida* based on 16s ribosomal RNA sequences (Gauthier et al. 1995).

Subsequent to the original outbreak, the bacterium was found to be responsible for smaller fish kills involving natural populations of striped bass in Chesapeake Bay (Paperna and Zwerner 1976) and in western Long Island Sound (Robohm 1979). The disease has been documented from cultured fish in the U.S. on only one occasion. In this instance, approximately 80% of the stocks of striped bass cultured in earthen brackish water ponds on the Alabama Gulf Coast were lost to the disease (Hawke et al. 1987). Although *P. damsela* subsp. *piscicida* has remained an important pathogen of cultured yellowtail (*Seriola quinqueradiata*) in Japan since the late 1960's (Kubota et

al. 1970, Egusa 1980), it has only recently emerged as a serious pathogen of cultured seabream (*Sparus aurata*) in Spain (Toranzo et al. 1991) and Italy (Ceschia et al. 1991) and in cultured sea bass (*Dicentrarchus labrax*) in France (Baudin-Laurencin et al. 1991) and Greece (Maragoudaki personal communication, 1993). The disease has recently been reported as causing serious economic losses in hybrid striped bass reared in ponds in Israel (Ariav, personal communication 1994). The importance of *P. damsela* subsp. *piscicida* as a pathogen of wild and farmed fish in Japan and in Europe has been established in several reviews (Toranzo et al. 1991, Kitao 1993, Thune et al. 1993 and Plumb 1994).

The only U.S. strains that have been subjected to phenotypic, antigenic and molecular characterization have been ATCC 17911 (Chesapeake Bay) and SB2-KK (Long Island Sound) both from the northern Atlantic Coast. This study is the first to characterize isolates affecting commercial aquaculture on the U.S. Gulf Coast. The objectives of this study were: 1) To compare representative Louisiana isolates with the only available U.S. strain, ATCC 17911 (Chesapeake Bay), and strains from Greece, Japan and Israel in various biochemical tests, tests for enzyme activity, antimicrobial susceptibility and plasmid profile; 2) To determine the % G + C of Louisiana strains 91-197 and 93-173 and ATCC strain 29687 (Japan), and 3) To determine if Louisiana isolates

could be detected in tissues from diseased fish with the Aquarapid-Pp rapid ELISA test (Bionor).

Materials and methods

Isolation of the etiological agent

Bacterial cultures were isolated from diseased hybrid striped bass voluntarily submitted to the Louisiana Aquatic Animal Diagnostic Laboratory (LAADL) by streaking samples of liver, kidney, and spleen on Brain Heart Infusion agar (BHIA; Difco Laboratories, Detroit, Michigan) supplemented with 2% NaCl or TSA II blood agar (5% sheep blood) (BBL, Cockeysville, Maryland) and incubating at 25°C for 48 hours. Isolated colonies were subcultured on BHIA + 2% NaCl.

Bacterial strains

The ten strains used in this study for comparison as well as their hosts, geographic origin and laboratory source are listed in Table 1. Bacterial strains were stored in an ultra-low freezer at -70°C in 500 µl aliquots of brain heart infusion broth (BHIB) supplemented with 2% NaCl and 20% glycerol.

Phenotypic analysis

Morphological, physiological, and biochemical tests were conducted according to methods outlined for halophilic organisms in the Manual of Clinical Microbiology (Farmer et al. 1985). Additional biochemical testing was done at 27°C

and 7 days incubation with the API 20E and 50CH systems (BioMerieux-Vitek, Inc., Hazelwood, Missouri). Bacteria were suspended in phenol red broth base (Difco) with 1% NaCl for inoculation of the 50CH system and in sterile saline (1% NaCl) for inoculation of the 20E system.

Rapid identification by ELISA

Fish sampled during outbreaks of photobacteriosis in Louisiana and fish experimentally infected with strain 91-197 were tested by ELISA for identity with the Aquarapid-Pp test kit using the manufacturers protocol (BioNor A/S, Skien, Norway). Briefly, spleens from infected hybrid striped bass were homogenized in buffer supplied with the kit and 7 drops of the homogenate placed in plastic spoons containing bound polyclonal antibodies to the European strain of *P. damsela* subsp. *piscicida*. Following a 15 minute incubation period the homogenate was washed off with water followed by buffer. Sheep anti-*P. damsela* subsp. *piscicida* conjugated with alkaline phosphatase was added to the spoon and allowed to bind to the captured antigen during a 15 minute incubation in the dark. Following 2x washes with water and buffer, three drops of substrate solution was added to the spoon. The presence of a red or pink color after a 15 minute incubation period was indicative of a positive test result. Positive and negative controls were run with each test.

Table 2. Strains of *Photobacterium damsela* subsp. *piscicida* used in molecular, biochemical and physical comparisons.

Strain	Donor ^a	Host	Origin
1079A	D. Maragoudaki	Sea Bass	Greece
EP94-001	T. Aoki	Yellowtail	Japan
93-320	R. Ariav	Hyb. striped bass	Israel
29687	ATCC	Yellowtail	Japan
17911	ATCC	White perch	Maryland
90-744	J. Hawke	Hyb. striped bass	Louisiana
91-197	J. Hawke	Hyb. striped bass	Louisiana
93-173	J. Hawke	Hyb. striped bass	Louisiana
94-069	J. Hawke	Hyb. striped bass	Louisiana
95-390	J. Hawke	Hyb. striped bass	Louisiana

a. ATCC, American Type Culture Collection, Rockville, Md; Dr. Takashi Aoki, Dept. of Aquatic Biosciences, Tokyo University of Fisheries, Tokyo, Japan; Dr. Raanan Ariav, Kibbutz Ma'agan Michael, Israel; Dr. Dimitri Maragoudaki, Zoological Institute, University of Leuven, Leuven, Belgium; John Hawke, Louisiana Aquatic Animal Diagnostic Laboratory, Dept. of Veterinary Microbiology and Parasitology, Louisiana State University, Baton Rouge, La. Louisiana strains are from different farm sites and different years.

Plasmid content

Strains used in this analysis are listed in Table 2. Cultures of the various isolates were started in 5 ml BHIB with 1% NaCl. Cultures were grown for 24 hours at 27°C in 17 X 100 mm glass test tubes placed in a roller apparatus (CEL-GRO, Lab Line Inc., Melrose Park, Illinois). Plasmid DNA was isolated by a modification of the alkaline lysis method of Birnboim and Doly (1979) as outlined in Sambrook et al. 1989. DNA samples (10-20 μ l) containing approximately 0.1 μ g DNA/ μ l were mixed with 5 μ l tracking dye and electrophoresed through 0.7% agarose gels in Tris-acetate (TAE) buffer at 10V for 18 hours in a horizontal apparatus (Biorad Laboratories, Richmond, California). Gels were stained with 0.5 mg/ml ethidium bromide for 30 minutes and destained with distilled water for two hours. A molecular weight standard, consisting of supercoiled DNA ranging from 2.067 to 16.21 kb (Gibco, BRL, Gaithersburg, Md), was included in all the electrophoresis runs for reference.

Antimicrobial susceptibility

Drug susceptibility was determined using the Kirby-Bauer disc diffusion method (Barry and Thornsberry 1980) with commercially available impregnated paper discs (Difco Inc., Detroit, Michigan). Vibriostatic agent 0/129 was dissolved in chloroform and added to sterile paper disc blanks (BBL Microbiology Systems, Cockeysville, Maryland).

Lawn cultures of strains to be tested were made on Mueller-Hinton agar (Difco) plates with 1% NaCl and five discs were spaced evenly per plate. The following chemotherapeutic agents ($\mu\text{g}/\text{disc}$) were employed: ampicillin (10), chloramphenicol (30), kanamycin (30), erythromycin (15), oxytetracycline (30), nitrofurantoin (300), sulfadimethoxine/ormetoprim (25), amoxicillin/clavulanic acid (30), and the vibriostatic agent O/129 (150).

Enzymic activity

The enzymic activity of various strains was determined using the API-ZYM system (BioMerieux-Vitek) incubated for 5 hours at 27°C. Inoculation, addition of reagents and reading of results were done according to the manufacturers protocol. Bacteria were suspended in 1.0% saline at a density equivalent to a McFarland #5 standard (Difco) prior to inoculating the strips.

Determination of midpoint denaturation temperature and G/C ratio

Growth of cultures

Starter cultures of *Photobacterium damsela* subsp. *piscicida* 91-197, 93-173 and ATCC 29687 were grown to log phase in 100 ml BHI broth with 2% NaCl. Two liter broth cultures were inoculated with 20 ml of the starter culture. A sample of the starter broth culture was streaked on a TSA II blood agar plate to check for purity. The cultures were grown for 16 hours at 25°C and 200 rpm on a rotary shaker

(New Brunswick Scientific, Edison, New Jersey) and cells harvested by centrifugation at 4000 x g for 30 minutes.

DNA extraction

DNA was extracted and purified by the chloroform-isoamyl alcohol method of Marmur (1961). The purity of each preparation was determined spectrophotometrically. A value of 1.8 for the ratio of optical density at 260 nm to optical density at 280 nm was used to demonstrate a lack of protein and RNA contamination.

DNA base composition

The midpoint denaturation temperature (T_m) of each DNA preparation was determined in a computerized Gilford Response II UV-VIS spectrophotometer with a thermocuvette, heating block, cooling water assembly and printer (Gilford Systems Inc., Oberlin, Ohio). Diluted DNA solutions were loaded into removable quartz micro-cuvettes and the instrument programmed for temperature increases of 1°C per minute. Printouts of the thermal denaturation profiles were produced and the mol% (G + C) calculated by the equation of Norgard and Bartel (1978).

$$\% \text{ G + C} = 100 \times \left(\frac{T_m}{50.2} \right) - .990$$

Results

Isolation of the etiologic agent

From fall of 1990 through fall of 1995, photobacteriosis was diagnosed from thirty-two submissions (cases) of diseased hybrid striped bass submitted to the Louisiana Aquatic Animal Diagnostic Laboratory (LAADL), School of Veterinary Medicine, Louisiana State University (Table 2). The bacterium was readily isolated from the spleen, liver and kidney of moribund specimens. Mortality rates during various outbreaks were variable depending on the use of antibiotic feeds and water quality conditions. In some instances mortality was less than 5% but in many units exceeded 90 % (Fernandez, personal communication, 1994). Control of the disease is complicated by the lack of antibiotics cleared for use with hybrid striped bass by the United States Food And Drug Administration (USFDA). In cases where Terramycin® or Romet® medicated feeds were used, an emergency compassionate INAD or a prescription was obtained. Effective therapy was further hindered by the emergence of Romet® and Terramycin® resistant strains of the pathogen in 1991 and 1994 following the use of medicated feeds.

Moribund fish collected during disease episodes typically swam listlessly near the surface and had no obvious external signs of disease except for a few individuals which exhibited slight hemorrhage around the

head and opercula. Internally, diseased fish had enlarged spleens that occasionally exhibited tiny white spots in the parenchyma. No other obvious internal clinical signs were noted.

Outbreaks of photobacteriosis typically occurred in the spring, although fall outbreaks were seen in 1990 and 1995. Affected fish ranged from 10 - 35 cm in length and mortality rates greater than 90% were noted in all size groups. Mortalities were observed at water temperatures between 17°C and 28°C and salinities between 3 and 17 ppt however, the majority of losses took place between 20 and 26°C and 6 and 10 ppt. Total mortality was greater than 50% of cultured stocks in 1991, 1994 and 1995 and included many valuable food size fish.

Phenotypic and biochemical characteristics of Louisiana isolates

Gram stains of small portions of kidney, spleen and blood revealed large numbers of Gram negative rods that exhibited bi-polar staining and pleomorphism. Streaks of samples of kidney, liver and spleen on 5% sheep blood TSAII agar (BBL) produced pure cultures of a single bacterial colony type after 48 hours incubation. Colonies were 2-3 mm in diameter, smooth, raised, entire, translucent, slightly viscid and non-hemolytic.

Table 3. *Photobacterium damsela* subsp. *piscicida* isolated from diseased hybrid striped bass submitted to the Louisiana Aquatic Animal Disease Laboratory (1990 - 1995).

Case #	Date	Water (°C)	Salinity (ppt)	Size (cm)	System type	Romet ^a
90-744	12-20-90	20	15	30	cage	S
91-197	03-28-91	24	08	22	pen	S
91-198	03-28-91	24	08	22	pen	S
91-216	04-12-91	24	10	18	pen	S
91-217	04-12-91	24	10	18	pen	S
91-218	04-12-91	24	10	18	pen	S
91-245	04-19-91	25	13	30	cage	S
91-276	04-23-91	22	06	22	pen	R
91-278	04-23-91	22	06	25	pen	R
91-289	04-30-91	23	06	25	cage	R
91-317	05-08-91	25	08	25	cage	R
91-318	05-08-91	25	08	25	cage	R
91-368	05-31-91	26	03	28	cage	R
92-196	06-05-92	28	NR ^b	25	cage	S
92-207	06-09-92	28	NR	25	pond	S
93-173	06-04-93	28	06	30	cage	S
94-056	03-31-94	18	07	30	pen	S
94-069	04-08-94	20	07	35	pen	S
94-070	04-08-94	20	07	15	pen	S
94-084	04-13-94	24	10	30	pen	S
94-085	04-13-94	24	10	30	pen	S

(table con'd)

Case #	Date	Water (°C)	Salinity (ppt)	Size (cm)	System type	Romet ^a
94-086	04-13-94	24	10	22	pen	S
94-088	04-13-94	24	10	30	pen	S
94-108	05-04-94	26	10	10	race ^c	S
94-109	05-04-94	26	10	15	pen	R
94-110	05-04-94	26	10	15	pen	R
94-118	05-05-94	26	10	10	race	S
94-130	05-05-94	26	10	15	pen	R
94-165	06-02-94	27	05	18	pen	R
94-425	12-02-94	17	15	25	pen	S
95-390	11-08-95	17	15	25	pen	S
95-393	11-10-95	17	17	25	pen	S

a. Romet susceptibility: S = susceptible, R = resistant.

b. no data reported with case.

c. raceway culture system

Preliminary tests showed the bacterial isolates to be non-motile Gram negative rods, 0.5 to 0.8 μm in width and 0.8 to 2.5 μm in length, catalase positive, oxidase positive, fermentative with no gas produced in glucose motility deeps and a K/A reaction with negative H_2S production in TSI slants. Bacterial cells were large and rod shaped in early log phase growth and tended to be smaller and more coccoid in older cultures. The bacteria

were sensitive to the vibriostatic agent 0/129 and required 0.5% salt in the medium for growth. All isolates produced a code number of 2005004 in the API 20E system. The results of phenotypic analysis of representative strains are included in Table 3. No variation among strains from different geographic locations was detected, although some strains were delayed or negative for maltose production.

Comparison of enzymatic activities

The results of analysis of the isolates in the API-ZYM system are reported in Table 4. All strains tested gave similar results with only slight differences in reaction intensity noted in tests for alkaline phosphatase, esterase and esterase-lipase. All Louisiana strains gave a similar profile for these three enzymes (code = 244), which differed from other strains. Low levels of cystine arylamidase were produced by strains 1079A, 93-320, ATCC 17911, LA93-173 and LA94-069, from varied locations.

Plasmid content

Louisiana strains of *P. damsela* subsp. *piscicida* were found to possess four identical plasmid bands with the exception of strain 90-744 which contained only a single plasmid (Figure 1). The Louisiana strains typically had two large plasmids that were not precisely sized due to a lack of large supercoiled molecular weight standards, but appeared to be in the 30 to 40 kb range. The two bands that were characteristic of all Louisiana strains tested

Table 4. Phenotypic characteristics of representative strains of *Photobacterium damsela* subsp. *piscicida*.

Test	LA strains ^a	<u>Strains</u> EP-001	1079A	93-320
Gram stain	-	-	-	-
Motility	-	-	-	-
Oxidase	+	+	+	+
Catalase	+	+	+	+
Voges-Proskauer	+	+	+	+
Indole production	-	-	-	-
Nitrate reduction	-	-	-	-
Citrate utilization	-	-	-	-
H ₂ S production	-	-	-	-
O/F glucose	O/F	O/F	O/F	O/F
Gas from glucose	-	-	-	-
Growth at 5°C	-	-	-	-
15°C	+	+	+	+
25°C	+	+	+	+
37°C	-	-	-	-
Growth in 0% NaCl	-	-	-	-
3% NaCl	+	+	+	+
5% NaCl	-	-	-	-

(table con'd)

Test	LA strains	EP-001	1079A	93-320
Arginine dihydrolase	+	+	+	+
Lysine decarboxylase	-	-	-	-
Ornithine decarboxylase	-	-	-	-
Tryptophan deaminase	-	-	-	-
β -galactosidase(ONPG)	-	-	-	-
Urease	-	-	-	-
Gelatinase	-	-	-	-
Hemolysis(5%sheep blood)	-	-	-	-
Acid production from:				
Glucose	+	+	+	+
Mannose	+	+	+	+
Galactose	+	+	+	+
Fructose	+	+	+	+
Ribose	+	+	+	+
N-Acetyl glucosamine	+	+	+	+
Maltose	V	(+)	(+)	(+)
Glycerol	(+)	(+)	(+)	(+)
Erythritol	-	-	-	-
Arabinose	-	-	-	-
Xylose	-	-	-	-
Adonitol	-	-	-	-
β -Methyl-xyloside	-	-	-	-
Sorbose	-	-	-	-

(table con'd)

Test	LA strains	EP-001	1079A	93-320
Rhamnose	-	-	-	-
Dulcitol	-	-	-	-
Inositol	-	-	-	-
Mannitol	-	-	-	-
Sorbitol	-	-	-	-
α -Methyl-D-mannoside	-	-	-	-
α -Methyl-D-glucoside	-	-	-	-
Amygdalin	-	-	-	-
Arbutin	-	-	-	-
Esculin	-	-	-	-
Salicin	-	-	-	-
Cellobiose	-	-	-	-
Lactose	-	-	-	-
Melibiose	-	-	-	-
Saccharose	-	-	-	-
Trehalose	-	-	-	-
Inulin	-	-	-	-
Melitzitose	-	-	-	-
Raffinose	-	-	-	-
Amidon	-	-	-	-
Glycogen	-	-	-	-
Xylitol	-	-	-	-
β -Gentiobiose	-	-	-	-

(table con't)

Test	LA strains	EP-001	1079A	93-320
Turanose	-	-	-	-
Lyxose	-	-	-	-
Tagatose	-	-	-	-
Fucose	-	-	-	-
Arabitol	-	-	-	-
Gluconate	-	-	-	-
2 or 5 cetogluconate	-	-	-	-

Symbols: +, positive reaction; -, negative reaction; (+), weak positive reaction; V, variable reaction; O, oxidative reaction; F, fermentative reaction.

with the exception of 90-744 were estimated to be 8.0 and 5.0 kb in size. No other strains from other geographic locations examined in the study or reported in the literature possess this same plasmid profile.

Strains from Japan, EP94-001 and ATCC 29687, possessed the typical bands of 3.5 and 5.0 kb reported from other pathogenic Japanese strains (Zhao and Aoki 1992). Strain ATCC 29687 possessed one high molecular weight plasmid and strain EP94-001 possessed two high molecular weight plasmids that could not be accurately sized (Figure 2).

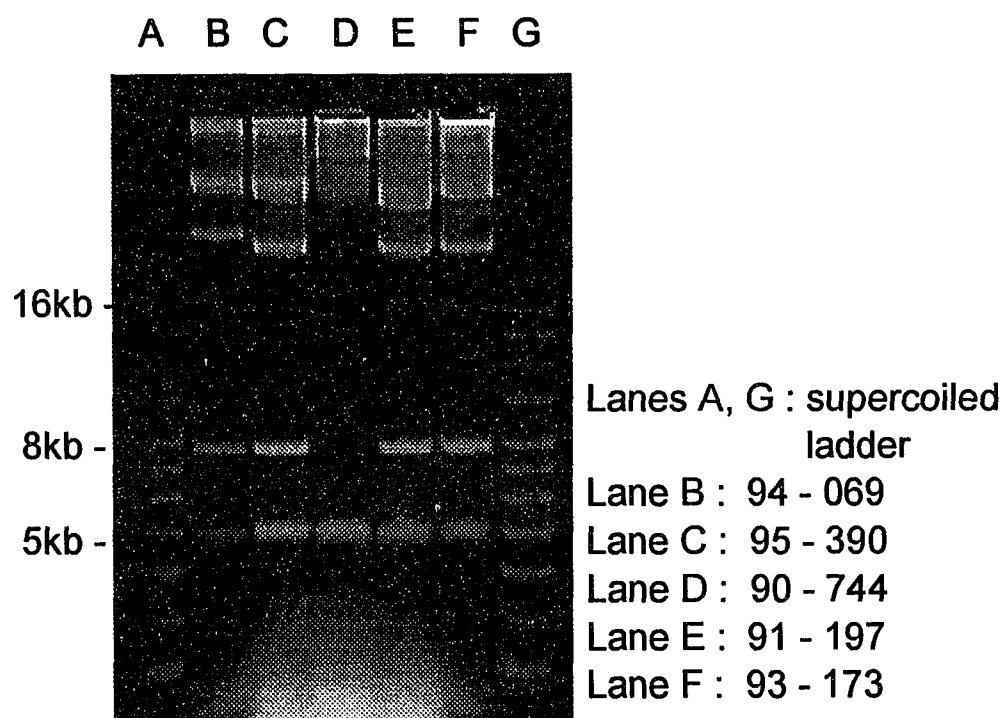


Figure 1. Agarose gel electrophoresis of plasmid minipreps showing the profile of Louisiana strains

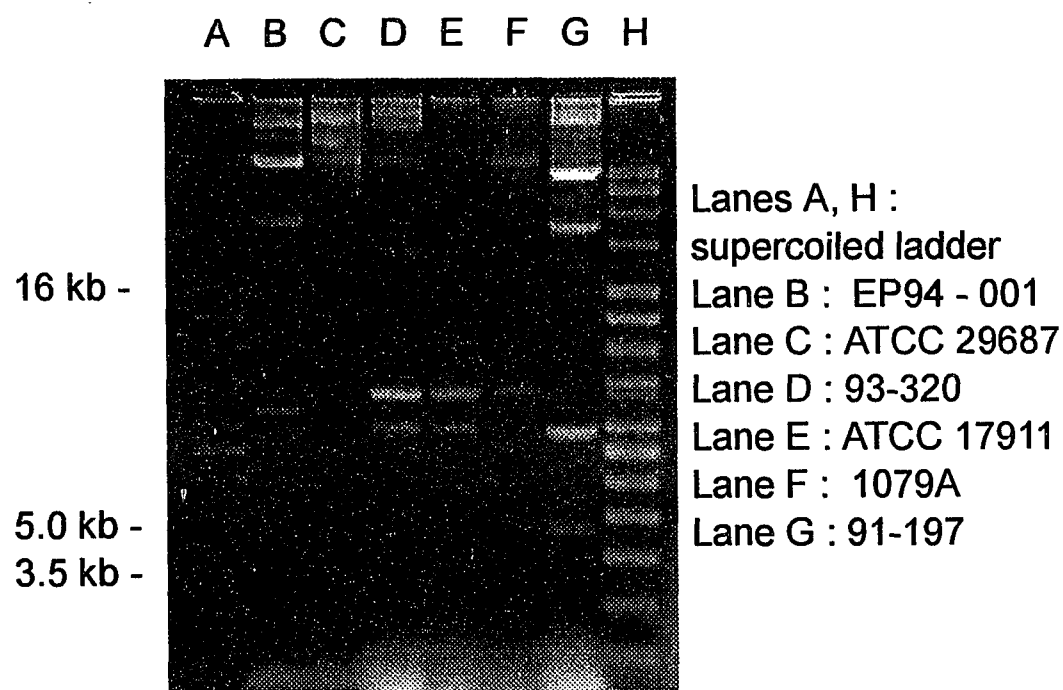


Figure 2. Agarose gel electrophoresis of plasmid minipreps showing the profile of representative strains

Table 5. Enzyme activities displayed by live cells of representative strains of *Photobacterium damsela* subsp. *piscicida*.

Strain	Enzymic Activties ^a								
	2	3	4	6	10	11	12	16	18
1079A	+	+	+	+	-	+	+	-	+
EP94-001	+	+	+	+	-	+	+	-	+
93-320	+	+	+	+	-	+	+	-	+
ATCC 29687	+	+	+	+	-	+	+	-	+
ATCC 17911	+	+	+	+	-	+	+	-	+
90-744	+	+	+	+	-	+	+	-	+
91-197	+	+	+	+	-	+	+	-	+
93-173	+	+	+	+	-	+	+	-	+
94-069	+	+	+	+	-	+	+	-	+
95-390	+	+	+	+	-	+	+	-	+

^a. Numbers match API code: 2, alkaline phosphatase; 3, esterase; 4, esterase-lipase; 6, leucine arylamidase; 10, α -chymotrypsin; 11, acid phosphatase; 12, phosphoamidase; 16, α -glucosidase; 18, *N*-acetyl- β -glucosaminidase.

(+, Positive response, -, Negative response).

All strains failed to produce lipase, valine arylamidase, cystine arylamidase, trypsin, β -galactosidase, β -glucuronidase, β -glucosidase, α -galactosidase, α -mannosidase and α -fucosidase.

Strains from Israel, Greece and ATCC 17911 exhibited a similar banding profile. Thus, plasmid profile is the only characteristic that can be used to differentiate Louisiana isolates from those from other geographic locations.

Antimicrobial susceptibility

Results of antimicrobial susceptibility are shown in Table 6. Three of the strains tested, 91-197 (Louisiana), 93-320 (Israel), and 1079A (Greece), showed uniform susceptibility to the antimicrobial agents used. The other two strains EP94-001 (Japan) and 91-278 (Louisiana) exhibited varying resistance patterns. Strain 91-197 was resistant to Romet[®] and oxytetracycline and strain EP94-001 was resistant to oxytetracycline, erythromycin, kanamycin and chloramphenicol.

Rapid identification by ELISA

Louisiana strain 91-197 produced a positive reaction with the Aquarapid-Pp rapid ELISA test kit. This indicates antigenic cross reactivity with the European strains used to produce the antibodies in the kit.

G/C ratio

Melting curves were plotted for the representative Louisiana strains 91-197, 93-173 and ATCC strain 29687. The melting temperature or midpoint of the thermal denaturation (T_m) was determined graphically on the Gilford Response II UV-VIS spectrophotometer to be 72°C for strains

Table 6. Antimicrobial susceptibility of representative strains of *Photobacterium damsela* subsp. *piscicida*.

Strain ^b	Antimicrobial Agent ^a							
	AM10	AMC30	C30	SOR25	T30	E15	K30	0/129
91-197	40 ^c	40	34	30	42	30	20	26
91-278	36	30	27	10*	10*	20	15	24
93-320	34	34	30	30	36	20	15	30
EP94-001	36	38	12*	26	10*	0*	0*	25
1079A	30	30	29	30	32	20	17	30

a. AM10 ampicillin 10 mcg, AMC30 amoxicillin/clavulanic acid 30 mcg, C30 chloramphenicol 30 mcg, SOR25 sulfadimethoxine/ormetoprim 25 mcg, T30 oxytetracycline 30 mcg, E15 erythromycin 15 mcg, K30 kanamycin 30mcg, 0/129 vibriostatic agent 150 mcg.

b. 91-197 and 91-278 (Louisiana), EP94-001 (Japan), 93-320 (Israel) and 1079A (Greece).

c. zone diameter in mm

* resistant

91-197 and ATCC 29687 and 71°C for strain 93-173 each strain. The DNA base composition, expressed as %(G + C), was calculated according to the formula of Norgard and Bartel 1978 to be 44.40 for strains 91-197 and ATCC 29687. Strain 93-173 had a % (G + C) calculated to be 42.43.

Discussion

This report is the first to document the occurrence of photobacteriosis in a commercially cultured species of fish in the U.S. The negative impact of photobacteriosis on the Louisiana mariculture industry is significant and could result in the failure of this potentially lucrative aquaculture industry.

Strains of *Photobacterium damsela* subsp. *piscicida* from various geographic locations, compared using molecular, phenotypic and ELISA techniques, were been shown to be almost identical. Several different investigators have come to the conclusion that this is a highly homogeneous bacterial species. The results of this study comparing Louisiana isolates with strains from Greece, Japan, Israel and Chesapeake Bay, USA, corroborate these findings in the areas of biochemical phenotype, enzymatic activities, % G + C, and antigenic cross reactivity. The overall homogeneity within the species makes *P. damsela* subsp. *piscicida* an excellent candidate for vaccine development.

The only area where variability exists is in the native plasmid profile and in antimicrobial susceptibility. The presence of two large plasmid bands of approximately 30 and 40 kb and two smaller plasmid bands corresponding to 8 and 5 kb are unique to Louisiana strains. The resistance to Romet® and Terramycin® by certain Louisiana strains was

the result of feeding medicated feeds containing these two drugs at different times in the same years. The resistance pattern seen in Japanese strain EP94-001 probably reflects the heavier use of drugs in Japanese aquaculture.

**CHAPTER III. EVENTS IN THE PATHOGENESIS OF PHOTOBACTERIOSIS
CAUSED BY THE BACTERIUM *PHOTOBACTERIUM DAMSELA* SUBSP.
PISCICIDA IN HYBRID STRIPED BASS (*MORONE SAXATILIS* X *MORONE*
CHRYSOPS): LIGHT MICROSCOPY AND BACTERIOLOGY**

Introduction

Photobacteriosis, caused by the bacterium *Photobacterium damsela* subsp. *piscicida* (formerly *Pasteurella piscicida*), is an emerging problem in the hybrid striped bass mariculture industry in Louisiana. From fall of 1990 through fall of 1995, photobacteriosis was diagnosed from thirty-two submissions of diseased hybrid striped bass to the Louisiana Aquatic Animal Diagnostic Laboratory (LAADL), School of Veterinary Medicine, Louisiana State University. The outbreaks of photobacteriosis occurred on mariculture farms located in the coastal parishes of Louisiana and resulted in losses of over one million dollars in 1991 and 1994 (Fernandez, personal communication, 1995). The importance of *P. damsela* subsp. *piscicida* as a pathogen of wild and farmed fish in Japan and in Europe has been established in several reviews (Toranzo et al. 1991, Kitao 1993, Thune et al. 1993 and Plumb 1994).

In natural outbreaks of photobacteriosis, external clinical signs are lacking except for pallor of the gills. Behavioral clinical signs include lethargy, increased respiratory rate and darkened pigmentation. In acute infections, few internal gross pathological signs are

observed except for reno/splenomegaly. In more chronic infections small white miliary lesions are present in the spleen (Hawke et al. 1987, Wolke 1975). The bacterium can be isolated from almost every tissue of moribund fish, indicative of a septicemia.

Effective treatment of photobacteriosis with medicated feed is contingent on an early diagnosis because sudden onset and explosive mortality are hallmarks of the disease. Mortality rates in some culture units reach 80% in only 10 days from the time of first detection of diseased fish (Chatry, personal communication, 1991). Currently there are no chemotherapeutics cleared for use on cultured populations of hybrid striped bass by the United States Food and Drug Administration (USFDA). An Investigational New Animal Drug (INAD) application submitted to the Center for Veterinary Medicine (CVM) USFDA permits treatment with Terramycin® or Romet®, two drugs that are cleared for use in catfish and salmonids. Development of resistant strains of the pathogen to both of these agents has occurred on multiple farms following medicated feed treatment (LAADL annual reports 1991-1995).

The above factors emphasize the need for new and better antimicrobial agents to treat photobacteriosis in hybrid striped bass and make vaccination a logical approach for the improved management of this disease. Before laboratory studies on vaccine and drug efficacy can be

conducted, a model for infection and pathogenesis is needed. Most studies of photobacteriosis in other fish species have relied on IP injection as the route of experimental infection, a method which does not mimic the natural route.

Consequently, objectives of this study were: 1) to determine if photobacteriosis could be induced experimentally by immersion exposure or oral exposure and if so, to calculate an LD₅₀ for each method; and, 2) to examine the sequential pathologic events involved in experimentally induced photobacteriosis in hybrid striped bass following immersion or oral exposure using bacteriological, histological, and light microscopic techniques.

Materials and methods

Experimental animals

Hybrid striped bass fingerlings (*Morone saxatilis* x *Morone chrysops*) ranging in size from 20 to 50 grams were obtained from two freshwater sources with no prior history of photobacteriosis (Lecompte Aquaculture Systems, Lecompte, Louisiana; Keo Fish Farm, Keo, Arkansas). Upon receiving the fish, a thorough bacteriological, parasitological and histological examination was conducted to insure that the fish population was in good health and free of *P. damsela* subsp. *piscicida* or any other infectious

disease. Prior to being used in infectivity or pathogenesis trials the specific pathogen free fish were acclimated for at least 30 days in a 728 gallon closed recirculating system maintained at 2 ppt salinity in the pathogen free Aquatic Pathobiology Laboratory at the LSU School of Veterinary Medicine. Fish were fed a 35% protein ration (SF Services Co., Little Rock, Arkansas) at the rate of 2% body weight per day, 5 days per week.

Water quality

Water quality was monitored daily, prior to and during each individual trial, to insure that parameters were within acceptable ranges. Tests for ammonia, nitrite and pH were conducted with a commercially available kit (Hach Chemical Co. Loveland, Colorado). Dissolved oxygen was determined with an oxygen meter (YSI model 58, Yellow Springs Instrument Co., Yellow Springs, Ohio). Salinity and temperature were determined with a salinity/conductivity meter, SCT model 33 (Yellow Springs Instrument Co.). Unless otherwise stated, the temperature was maintained at 22°C and salinity at 10 ppt for all infectivity and pathogenicity experiments.

Infectivity and pathogenesis trials

Trial #1 Infection by immersion

An initial infectivity and virulence trial was conducted to determine if strains of *P. damsela* subsp. *piscicida* isolated from natural outbreaks on Louisiana fish

farms were pathogenic for hybrid striped bass by immersion (water-borne) exposure. A representative Louisiana strain, LA91-197, stored in Brain Heart Infusion broth (BHIB, Difco) with 20% glycerol in an ultra low freezer at -70°C was recovered in 5 ml BHIB + 2% NaCl. A 100 ml culture was grown in BHIB + 2% NaCl in an Erlenmeyer flask in a controlled environment incubator shaker (New Brunswick Scientific Co., Edison, New Jersey) at 200 rpm for 24 hours. The broth culture was centrifuged at $4000 \times g$ for 20 minutes and the pelleted cells resuspended in sterile phosphate buffered saline (PBS). The number of cells in the LA91-197 suspension was calculated to be 2.65×10^9 colony forming units (CFU)/ml by the drop plate method as follows. Briefly, ten-fold serial dilutions of the original suspension were made in sterile PBS. Three replicate 20 μl drops of each dilution were placed onto tryptic soy agar plates with 5% sheep blood (TSA II, BBL, Cockeysville, Maryland). The dilution that produced countable colonies was used to back calculate the number of CFU/ml in the original suspension. Three 20 liter static tanks, containing five fish each, received one ml of the bacterial suspension. Biological filtration was supplied by a single 500 ml air-lift bead filter located within each tank. The infectious dose of LA91-197 was 132,500 CFU/ml by indefinite immersion exposure.

Trial #2 - Determination of LD₅₀

Prior to initiating trial 2, hybrid striped bass fingerlings were exposed to *P. damsela* subsp. *piscicida* strain LA91-197 by immersion and the bacterium reisolated three times to confirm virulence. To determine the LD₅₀ by immersion exposure, hybrid striped bass (mean weight 51 grams) were exposed to the bacterium in 20 liter static tank systems using the same filtration system as trial 1. To determine an oral LD₅₀, hybrid striped bass (56 grams mean weight), were exposed to the bacterium by oral intubation using 1 mm diameter plastic tubing attached to a 26 gauge syringe needle. Tanks were stocked with 3 fish each and the fish were acclimated to the systems for one week prior to experimental infection. One ml of strain LA91-197 was inoculated into 50 ml BHIB + 2% NaCl and the cells harvested after 18 hours of growth in an incubator shaker at 25°C and 200 rpm. Ten-fold dilutions of the bacterial culture were made in 4.5 ml sterile PBS blanks and 1 ml of each dilution was added to the appropriate system for the immersion challenge and 0.1 ml of each dilution was given to each fish by intragastric intubation in the oral challenge. The number of bacteria per ml present in each suspension was determined by colony counts on TSA II blood agar plates using the drop plate method. Two tanks were inoculated with each dilution and mortality was recorded daily. The exposure (dose) ranged from 125 to

125,000 CFU/ml of tank water in the immersion challenge and 150 to 1.5×10^6 CFU/fish in the oral challenge. The trial was continued until no mortalities were recorded for ten days. All surviving fish were necropsied at the conclusion of the trial and tissues were cultured for the presence of *P. damsela* subsp. *piscicida*. The LD_{50} was calculated by the method of Reed and Muench (1938).

Trial #3. Bacteriology and histopathology (Low Dose):

Four fish, mean weight 58 grams, were stocked in each of ten 70 liter recirculating systems and allowed to acclimate for one week. The systems were composed of two 35 liter tanks with water recirculated between the two tanks by means of a 1/2 inch PVC air-lift pump (pumping rate 3.6 liters per minute) and a 1 inch PVC siphon tube return. Fish were held in one tank and a combination of an air-lift sponge filter and a 500 ml air-lift bead filter was used for biological filtration in the second tank. Fish were exposed to 5700 CFU/ml of a suspension of strain LA91-197 by direct addition of 1 ml of a 10^{-1} dilution of bacterial suspension (550 Klett units) to each system. The number of bacteria in the inoculum was determined by the drop plate method. Three fish were sampled from the control tanks at the start of the trial and three fish were randomly sampled from the infected systems every 24 hours post-infection until fish began to exhibit abnormal behavior. At this time at least two fish exhibiting

clinical signs were included in each sample. Sampled fish were euthanized in 0.5% MS-222 (3-aminobenzoic acid, methanesulfonate salt); (Sigma Chemical Co., St. Louis, Missouri) prior to necropsy. A 1 ml blood sample was drawn from the caudal vein of each fish in a 2 ml draw vacutainer containing 7.5% EDTA (K_3) as an anticoagulant (Vacutainer, Becton Dickenson Co.) and the tube placed on ice until it was diluted. Each fish was aseptically necropsied and small (2 mm^3) sections of liver, trunk kidney and spleen were removed and placed in individual pre-weighed sterile eppendorf tubes containing $1000\text{ }\mu\text{l}$ of sterile PBS at 4°C . Tissue samples contained in the tubes were weighed and homogenized. Serial ten-fold dilutions of the homogenized tissues and blood were made in cold sterile PBS. Bacteria in the tissues and blood were enumerated by the drop plate method and were reported as CFU per gram of tissue or per ml of blood. The remaining tissues of each fish were fixed in neutral buffered 10% formalin for histopathology. The liver, head kidney, trunk kidney, heart, intestine, gills and skin were processed, embedded in paraffin and sectioned at $5\text{--}7\text{ }\mu\text{M}$ in compliance with accepted histological procedures (Humason, 1972). Heads were decalcified in either 10% EDTA for one week or 10% formic acid for 3 days prior to standard processing. Cross sections of the head containing sections of the brain and olfactory lamellae were embedded in paraffin and subjected to standard

processing. The paraffin sections were stained with Harris' hematoxylin and eosin (H&E) (Humason, 1972). Tissues, cells and blood cells were identified according to Groman (1982).

Trial #4. Bacteriology and histopathology (High Dose):

Fifty fish, mean weight 111.2 g, were stocked into a single, 140 gallon (530 liter) recirculating system, with a plastic bead style biological filter (Armant Aquaculture model no. BBF-2). Ten fish were acclimated in the system for two weeks and the remaining forty fish were added after the filter had become seeded with nitrifying bacteria. Following additional acclimation for three days, the fish were exposed to 13,000 CFU/ml of a suspension of strain LA91-197 by direct addition of 10 ml of a bacterial suspension containing 7.0×10^8 cfu/ml (400 Klett units). The fish were exposed to the higher dose to ensure uniformity of infection. Four fish were removed prior to infection to serve as controls and four fish were removed daily at 24 hour intervals post-inoculation. After the second day only moribund specimens were collected. No fish remained alive after 96 hours. Methods of blood and tissue collection, bacterial enumeration in tissues and blood and tissue fixation were the same as in trial 1.

Trial #5. Early gill histopathology

This study was proposed after trials 3 and 4 implicated the gill as the site of entry following water-

borne exposure. Four hybrid striped bass (mean weight 50 grams) were exposed to 2.7×10^7 CFU/ml of strain LA91-197 in 3.7 liters of water for 30 minutes then placed in clean water for 30 minutes to purge the gills of excess bacteria. The fish were transferred to a 70 liter recirculating system where they were maintained at 22°C and 10 ppt salinity. Fish were sacrificed at 24 and 48 hours post exposure. The gill tissues were fixed for 6 hours in cold 1.25% gluteraldehyde + 2% formaldehyde in 0.1 M cacodylate buffer (pH 7.2). Following fixation the tissues were rinsed and stored in 0.1M sodium cacodylate buffer (pH 7.2). Semi-thin sections (1 μ m thick) were cut on a Reichert Om U3 microtome (Reichert, Austria) and stained with 1% toluidine blue and basic fuchsin.

Statistical evaluation

Logarithmically transformed bacterial counts from each tissue and each time period in trials 3 and 4 were analyzed using the general linear model for analysis of variance (ANOVA) and are shown as the mean \pm the standard deviation. Comparison of means was done on a daily basis by least significant difference (LSD) using an analytical software package (Statistix[®] version 4.1, Tallahassee, Florida). A significance level of $\alpha = 0.05$ was used.

Results

Water quality

Water quality parameters during the trials ranged as follows:

Salinity	10.0 ppt
Ammonia (NH ₄)	0.4 - 1.6 ppm
Nitrite (NO ₂)	0.1 - 2.5 ppm
pH	8.0 - 8.5
Dissolved oxygen	6.5 - 7.5 ppm
Temperature	22.0 - 23.5 °C

Trial 1

A representative strain from outbreaks of photobacteriosis in Louisiana (LA91-197) was found to be virulent for hybrid striped bass in the laboratory by immersion. Fish sampled at 24 and 48 hours post-infection (PI) cultured positive for *P. damsela* subsp. *piscicida* and at 96 hours PI all fish had died from the disease.

Trial 2

The immersion LD₅₀ was calculated to be 687 CFU/ml. Fish in the 125,000/ml dose died 72 hours PI. Fish exposed to 12,500 CFU/ml began dying 96 hours after exposure and were all dead by day 6 PI. The last dilution at which mortality occurred was the 10⁻², containing 1250 CFU/ml with deaths being recorded from the fifth through the tenth day. No fish died of photobacteriosis from day 11 PI until the

experiment was terminated on day 21. Surviving fish were necropsied at the conclusion of the trial and strain 91-197 was not isolated from the liver, kidney, brain, blood or intestine of any survivor nor was there any evidence of chronic disease in any survivor. No fish died in the oral challenge at any dosage.

Trial 3

Clinical signs

External clinical signs were minimal throughout the course of infection. Mild hyperemia in the vicinity of the nares and at the base of the dorsal and pectoral fins was noted on day 3 PI. The only additional clinical signs observed in moribund fish were; increased opercular rate, lethargic behavior, lack of pigment regulation and pallor of the gill filaments. Strain 91-197 was not isolated from experimentally infected fish and no histopathological changes were noted until 72 hours PI when all tissues cultured positive. The infection can be classified as an acute generalized septicemia with almost all of the organs and tissues involved in the latter stages of the disease. The first moribund specimens were collected on day 5 and by day 7 PI all fish in the challenge systems were dead.

Histopathology

Gills

The earliest lesions were seen in the gills on day 3 PI. These lesions included rare focal areas of necrosis

and edema at the basal area of the secondary lamellae. There were occasional bacteria observed free in the blood or within macrophages. Moribund fish sampled on day 5 and 6 PI had extensive necrosis of the gill lamellae with edema and loss of epithelium (Figure 3). There were degenerative changes in epithelial cells and chloride cells characterized by nuclear pyknosis and karyorrhexis. Capillaries in the gill filaments and sinusoids of the secondary lamellae were occluded with masses of bacterial cells (Figure 4).

Spleen

The earliest evidence of disease in the spleen was observed on day 3 PI. Pigmented macrophages containing bacteria were observed in the area of melanomacrophage centers (Figures 5 and 6). From day 4 until death, the spleen exhibited sites of acute multifocal focal necrosis associated with numerous bacterial colonies in the area of the splenic ellipsoids (Figure 7). In some areas the bacterial colonies appeared to arise from the engorgement of or replication of bacteria within macrophages and subsequent cell death (Figure 8). Melano-macrophage centers of the spleens of moribund fish collected on days 5 and 6 PI seemed less organized and depleted of cells. Very little inflammatory response was noted during any stage of the infection.

Figure 3: Photomicrograph of gill lamellae from a moribund specimen 6 days post infection with *P. damsela* subsp. *piscicida*. Capillaries (c) supplying blood to the secondary lamellae are occluded with bacteria (b) (H&E, X 400).

Figure 4: Photomicrograph of gill lamellae from a moribund specimen 6 days post infection with *P. damsela* subsp. *piscicida*. Degenerative changes are seen in the primary and secondary lamellae including edema and loss of epithelium (H&E X 400).

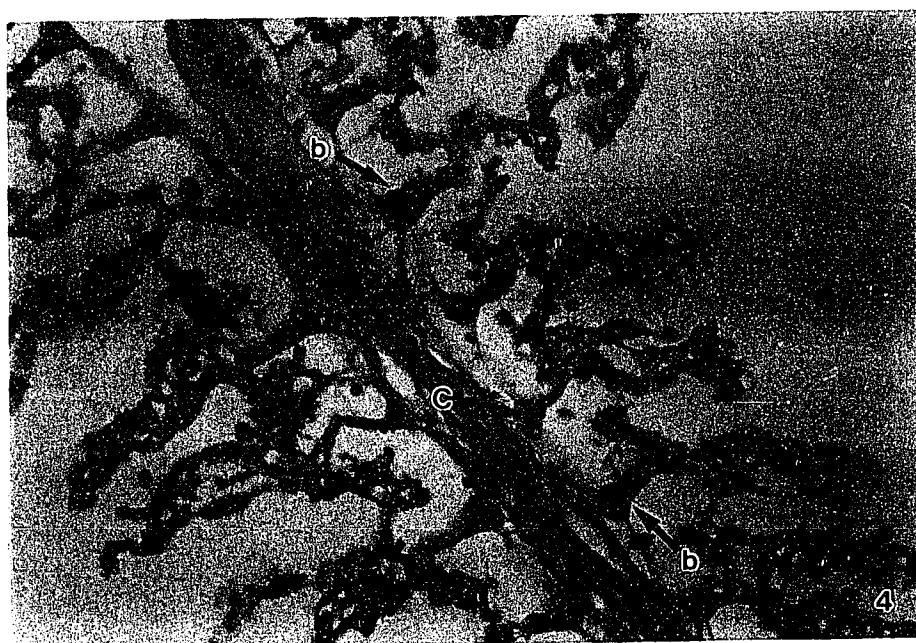


Figure 5: Splenic macrophages (m) containing phagocytized bacteria 3 days post infection (H&E X 1600)

Figure 6: Splenic macrophages (m) containing many bacterial cells 4 days post infection (H&E X 1600)

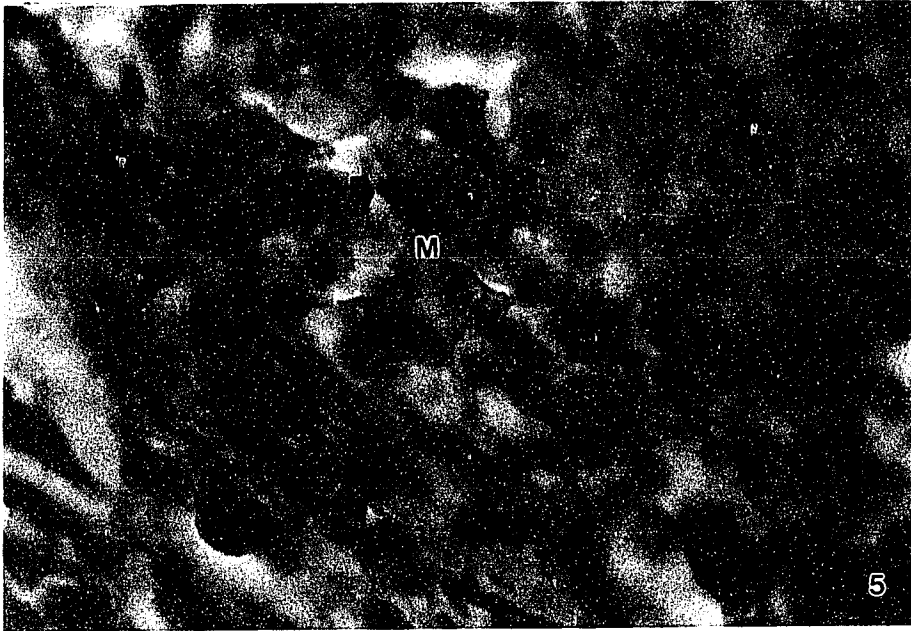
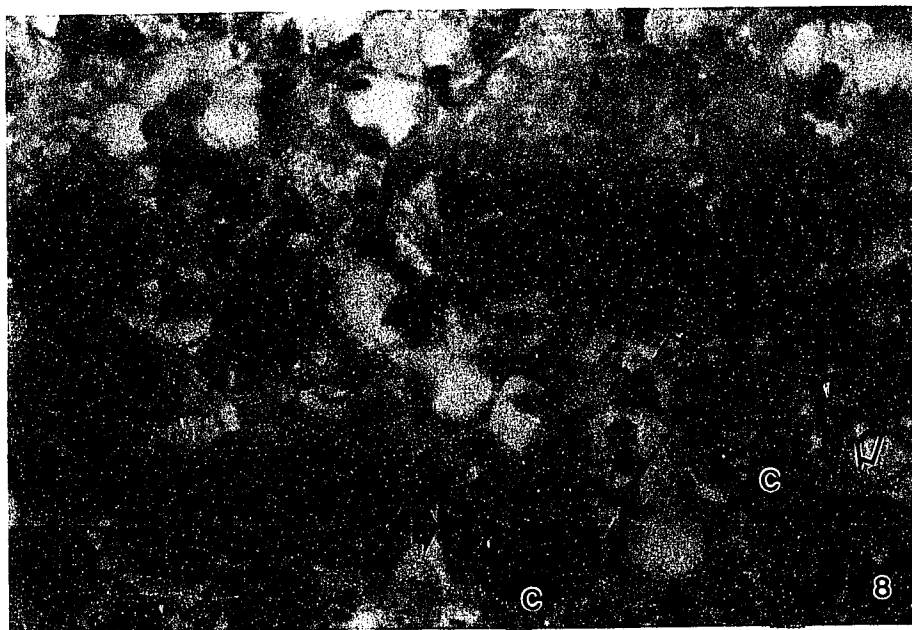
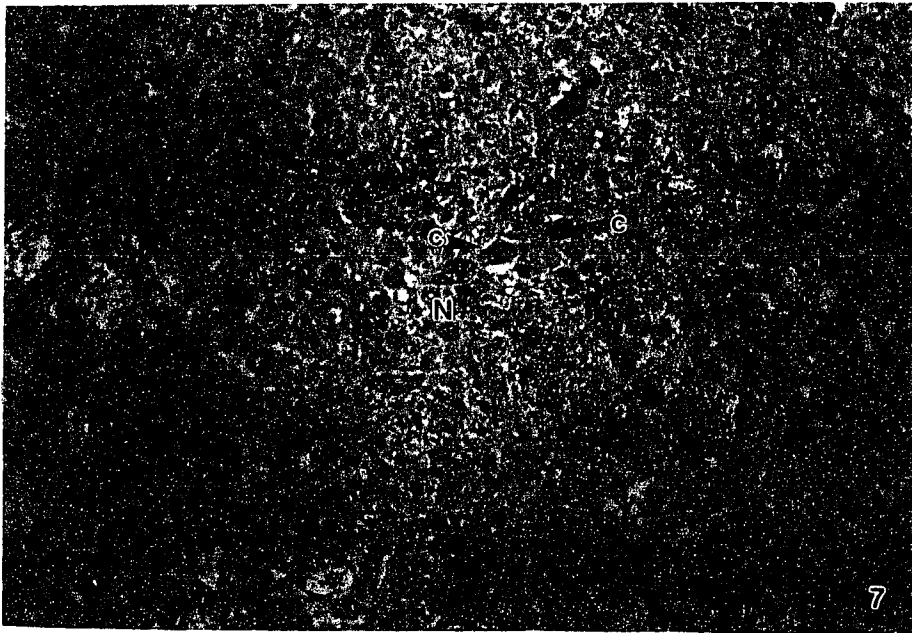


Figure 7: Focal necrosis (n) in the spleen associated with numerous colonies (c) of *P. damsela* subsp. *piscicida* (H&E X 100)

Figure 8: Spherical bacterial colonies (c) in the area of the splenic ellipsoids possibly resulting from the death of macrophages engorged with bacteria (H&E X 1600).



Kidney

Early lesions were not detected in the trunk kidney. An interstitial nephritis was noted in limited areas of the trunk kidney with bacterial cells visible within macrophages by day 4 PI. The trunk kidney was severely affected by day 5 PI with widespread renal tubular and glomerular necrosis. Lesions in the head kidney were similar to those noted in the spleen, with macrophages containing bacteria and bacterial colonies becoming numerous on day 3 and 4 PI. By day 5 PI, extensive acute multifocal necrosis of the hematopoietic tissue had occurred. In general, very little inflammatory response was noted in the kidney tissues.

Liver

The liver was the last organ to become colonized with bacteria and showed the fewest pathologic changes. After day 4 PI there was moderate acute multifocal necrosis of the hepatocytes with prominent karyorrhexis. Bacteria were visible in the sinusoids and within the hepatic vessels in moribund specimens. Occasionally, the pancreatic tissue surrounding the vessels exhibited acute multifocal necrosis with a reduction in size of zymogen granules. Inflammatory cellular accumulations were absent in the liver during any stage of the infection.

Other tissues and organs

There were no early lesions detected in the olfactory lamellae, brain, intestine, heart, or skin. High numbers of bacteria and associated lesions were found in almost all tissues of moribund fish.

Bacteriology

The mean levels of bacteria \pm the standard deviation detected in sequential analysis of tissues and blood are listed in Table 7 and are graphically depicted in Figure 9. Briefly, *P. damsela* subsp. *piscicida* was first detected in all tissues sampled at 72 hours post infection. The highest cell count was detected in the spleen, with the next highest levels in the kidney, liver and blood respectively. Bacterial numbers increased daily in all the tissues until the termination of the experiment at which time the spleen was found to contain the highest number of CFU/gram at 5.7×10^9 . The other tissues were clustered slightly above 2.0×10^8 CFU/gram or ml. Controls were negative for bacteria and no mortality occurred.

Trial 4

Clinical signs

The clinical signs exhibited by fish in this trial were identical to those observed in Trial 3, however, the onset of disease was much faster and the uniformity of infection among fish was greater because of the higher

Table 7. Logarithmic transformation of mean daily CFU/gram of *Photobacterium damsela* subsp. *piscicida* isolated from the liver, kidney and spleen or CFU/ml of blood with standard deviations following immersion infection with a low dose (6000 CFU/ml). Means not significantly different are indicated by the same letter.

Tissue	Day				
	1-2	3	4	5	6
Liver	0	2.6(±2.3)bc	4.7(±0.1)c	7.0(±1.6)bc	8.3(±0.5)b
Kidney	0	3.9(±3.5)ab	5.9(±0.3)b	8.1(±0.8)bc	8.5(±0.2)ab
Spleen	0	5.9(±1.6)a	8.4(±0.5)a	8.5(±0.3)a	9.6(±0.4)a
Blood	0	1.2(±2.2)c	4.4(±0.2)c	6.1(±0.4)c	7.7(±1.0)b

infectious dose. The first moribund specimen was collected on day 3 PI and by day 5 no fish remained alive.

Histopathology

Gills

The presence of focal areas of necrosis in the gills in conjunction with bacterial colonization in the first 48 hours indicated that the gills are the primary site of bacterial entry into the fish. This finding prompted another study to look specifically at the gills following exposure to high doses of the bacterium.

Spleen

Lesions in the spleen were identical to those observed in Trial 3 however they occurred much earlier. Bacteria were observed within splenic macrophages within the first 24 hours PI and colonizing the spleen as early as day 2 PI.

Acute multifocal necrosis associated with numerous bacterial colonies occurred by day 3 PI.

Kidney

Lesions in the head and trunk kidney were similar to those observed in Trial 3 except colonization and necrosis occurred as early as day 2 PI in the head kidney and day 3 PI in the trunk kidney.

Liver

The liver was colonized much later than other organs and did not show pathological changes until the third day post infection. The lesions were the same as those reported in Trial 3.

Other organs and tissues

As in the previous study, no early involvement was seen in the olfactory lamellae, intestine, or other tissues. As early as day 3 PI, septicemia had developed in most fish and high bacterial numbers were detected in most tissues sampled.

Bacteriology

The levels of bacteria detected in sequential analysis of tissues and blood are listed in Table 8 and are graphically depicted in Figure 10. Bacteria were detected in the spleen of one fish at 24 hours post infection and were present in higher numbers in the spleen at each sampling period. The liver was the last organ to be colonized and had the lowest counts throughout the study.

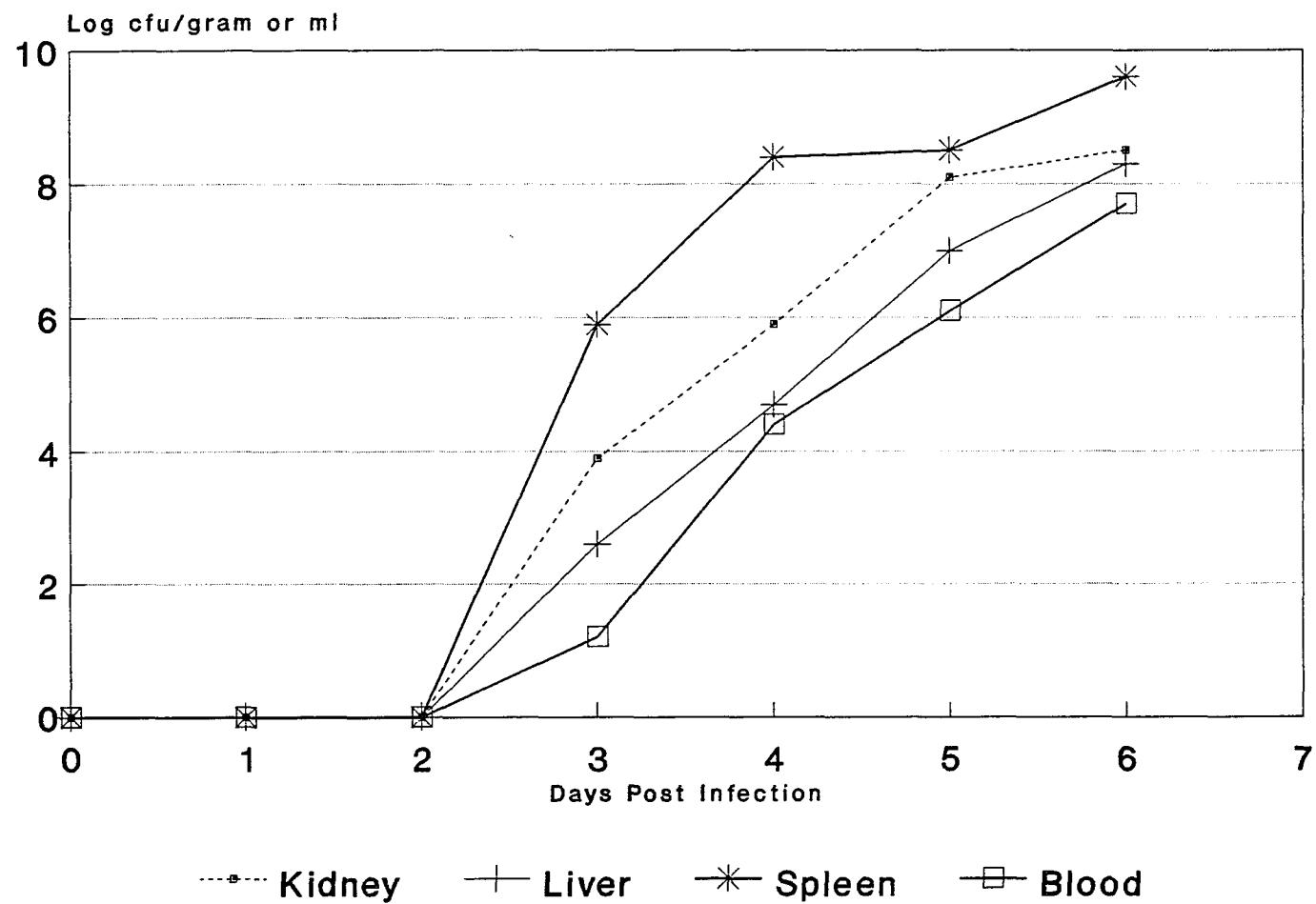


Figure 9. Daily bacterial counts (CFU/g) from liver, spleen, kidney and (CFU/ml) from blood of hybrid striped bass infected by immersion with a low dose (6000 CFU/ml) of *Photobacterium damsela* subsp. *piscicida*

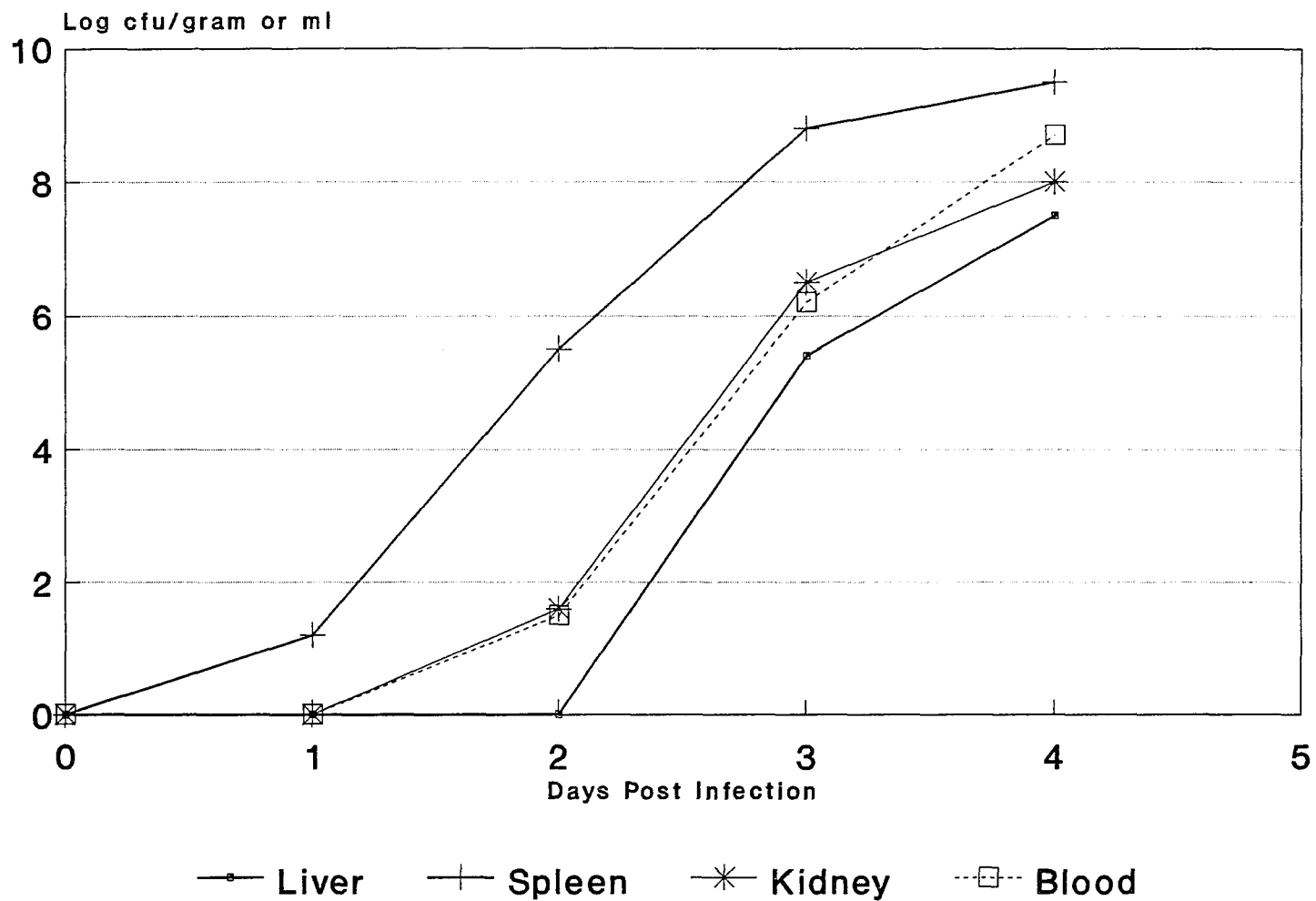


Figure 10. Daily bacterial counts (CFU/g) from liver, spleen, kidney, and (CFU/ml) from the blood of hybrid striped bass infected by immersion with a high dose (13,000 CFU/ml) of *Photobacterium damsela* subsp. *piscicida*

Trial 5**Gill histopathology**

Photobacterium damsela subsp. *piscicida* was found to penetrate the gill epithelium and establish infection in the gill lamellae at 24 hours post immersion. Normal gills are shown in figure 11. The bacteria were found extracellularly beneath the epithelial cells and intracellularly within macrophages and chloride cells in the secondary lamellae and lamellar troughs (Figure 12). In association with bacterial colonization there was acute multifocal necrosis of various cell types characterized by nuclear pyknosis and karyorrhexis. Areas of edema associated with invasion and multiplication of bacterial cells were noted in the secondary lamellae at 24 hours PI (Figure 13). Granular eosinophils were often seen accumulating in the secondary lamellae, where bacterial cells were numerous (Figure 14). At 48 hours PI there were widespread areas of necrosis of various cell types in the primary and secondary lamellae however, bacterial colonies were no longer visible.

Discussion

Infectivity trials with *Photobacterium damsela* subsp. *piscicida* and hybrid striped bass (*Morone saxatilis* X *Morone chrysops*) revealed that this bacterium ranks as one of the most virulent bacterial fish pathogens, with an

Table 8. Logarithmic transformation of mean daily CFU/gram of *Photobacterium damsela* subsp. *piscicida* isolated from the liver, kidney, and spleen or CFU/ml of blood with standard deviations following immersion infection with a high dose (13,000 CFU/ml). Means not significantly different are indicated by the same letter.

Tissue	Day			
	1	2	3	4
Liver	0	0 b	5.4(±0.5)b	7.5(±0.5)c
Kidney	0	1.6(±1.8)b	6.5(±1.4)b	8.0(±0.6)bc
Spleen	1.2*	5.5(±1.1)a	8.8(±0.3)a	9.5(±0.4)a
Blood	0	1.5(±1.1)b	6.2(±0.8)b	8.7(±0.6)b

* only one fish cultured positive

immersion LD₅₀ less than 1250 cells/ml. Infection could not be established by intragastric (oral) inoculation. The LD₅₀ was previously determined to be 4.0 X 10⁴ cells per fish by IP injection in juvenile striped bass by Robohm (1983). No previous studies have been done on oral or immersion exposure with striped bass or hybrid striped bass.

The sequential pathology and bacteriology following immersion exposure revealed that at low infectious doses (6000/ml) the bacterium was first detected in the spleen and gills at 3 days PI. At the higher infectious doses the gills appear to be the site of entry of the organism. The gills were not sampled during the sequential bacteriology

Figure 11. Normal gill lamellae

Figure 12. Cells of *P. damsela* subsp. *piscicida* shown intracellularly within the secundary lamellae and the lamellar trough

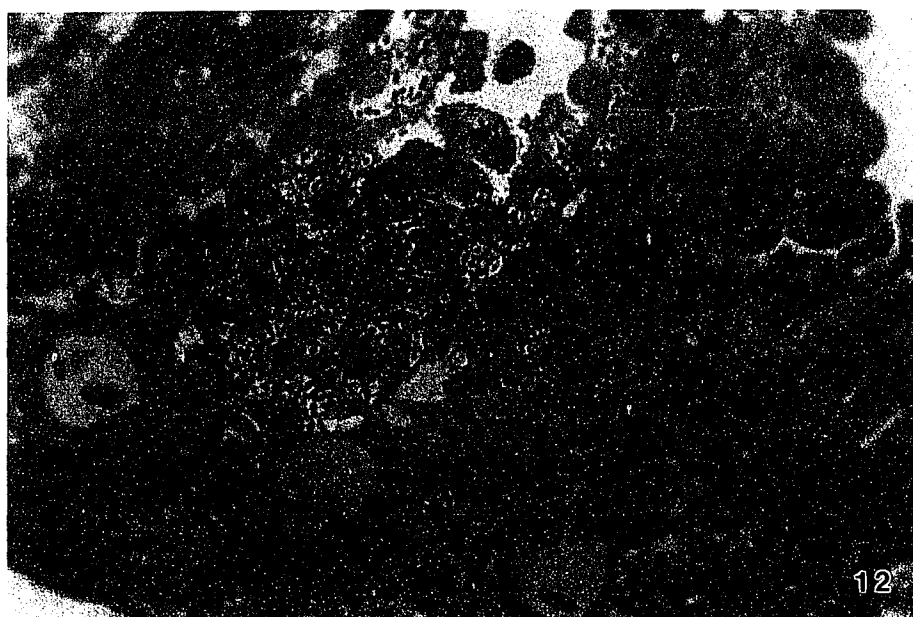
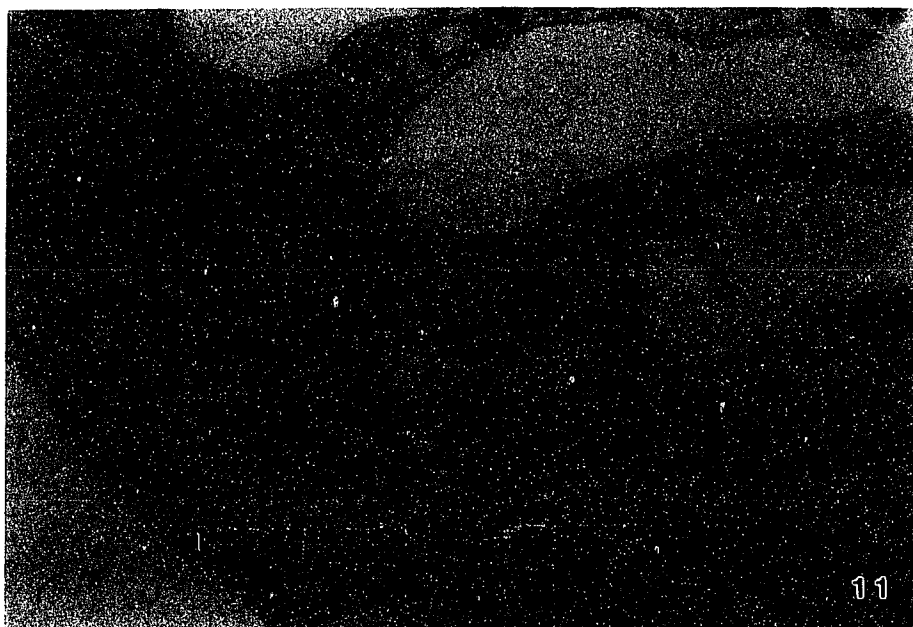


Figure 13. Areas of edema in the secondary lamellae associated with invasion and multiplication of bacterial cells 24 hours PI

Figure 14. Granular eosinophils associated with high numbers of bacterial cells in the secondary lamellae 24 hours PI



study because of the problem with contaminating organisms in the system water. Future studies could include the gills if a procedure for decontaminating the surface is developed. It appears that following penetration of the epithelium, a period of replication in the gill lamellae takes place. The bacterium either leaves the gill tissue intracellularly in macrophages as free cells in the blood and moves via the circulatory system to the head kidney, trunk kidney and the spleen. Based on the route of blood flow through the circulatory system the liver is the last organ of those sampled to receive infectious agents transported via the blood from the gills. Macrophage rich organs like the spleen and kidney phagocytose bacteria transported there by the blood and these bacteria-laden macrophages are particularly obvious in histological sections of the spleen. Proposed intracellular survival of phagocytosed bacteria within macrophages could allow their numbers to build to the high levels noted during infection. This may not be the case, however, because macrophages obtained from rainbow trout, sea bass, and gilthead sea bream evaluated in an *in vitro* assay were found to kill both virulent and avirulent strains of *P. damsela* subsp. *piscicida* (Skarmeta et al. 1995). The ability of hybrid striped bass macrophages to kill this pathogen has not been assessed, but if they are also capable of killing the pathogen,

bacterial numbers detected in the tissues may be the result of the ability of the organism to survive and proliferate in the blood which results in continued entrapment in the spleen by macrophages. The survival of the pathogen in the tissues could be the result of the macrophage being overwhelmed by large numbers of bacterial cells carried in the blood. If this is the case, the siderophore would be an important virulence factor for *P. damsela* subsp. *piscicida*.

Statistical analysis clearly shows that the spleen is an early target of infection and contains a significantly higher number of bacteria than the other tissues throughout the course of the infection, both in the low dose and high dose immersion infection studies. The liver is the last organ to be colonized and at most sample periods contains the lowest number of bacteria.

Finally, unrestricted replication of the organism in the blood due to resistance to serum killing and replication in the tissues may result in release of enough bacterial toxins to cause localized tissue damage, particularly in the gills. Following injection of purified ECP into gilthead sea bream Noya et al. (1995) found that there was an absence of lesions adjacent to the injection site and in the spleen. The authors felt this lack of tissue-destructive capability was due to the low proteolytic activity of the ECP. Epithelial and chloride

cells of the gills suffered degenerative changes after the injection of ECP perhaps indicating greater sensitivity of these cells to the bacterial toxins. Alternatively, the tissue damage in the gills could be due to ischemia associated with blockage of blood supply by the large numbers of bacteria that accumulate in the gill capillaries.

The cause of death in hybrid striped bass infected with *P. damsela* subsp. *piscicida* is probably respiratory failure resulting from the combination of necrosis of the gill epithelium and supporting tissues and the congestion of the sinusoids and capillaries of the secondary lamellae of the gills with bacterial cells. This is consistent with the finding of Kubota et al. (1970) that death of yellowtail is by suffocation resulting from bacterial embolism or macrophage infiltration in the gill lamellae.

**CHAPTER IV. EVALUATION OF A SIDEROPHORE BIOSYNTHETIC
MUTANT OF THE MARINE BACTERIUM *PHOTOBACTERIUM DAMSELA*
SUBSP. *PISCICIDA* GENERATED BY TRANSPOSITIONAL MUTAGENESIS**

Introduction

The term *siderophore* was coined by Lankford (1973) in reference to the low molecular weight, high affinity iron ligands produced by microorganisms in order to survive in an aerobic environment where iron is virtually insoluble at biological pH and limited in its availability (Schwyn and Neilands, 1987). Iron is an essential element, important in the metabolism of aerobic and anaerobic organisms. It is a component in the array of proteins composing the electron transport chain designed to permit efficient reduction of O₂ and high yields of ATP. An iron-containing reductase enzyme is involved in the generation of 2'-deoxyribonucleotides needed for DNA synthesis (Neilands, 1990).

Iron availability is also a critical factor in the pathogenicity of microorganisms. The amount of free iron available to the pathogen within the host is extremely low. Most extracellular iron is bound to proteins such as transferrin or lactoferrin present in the serum or found intracellularly in molecules such as hemoglobin, ferritin, hemosiderin and free heme (Griffiths, 1987). Microorganisms must be able to adapt to this iron-restricted environment and develop mechanisms of assimilating iron

bound to these compounds in order to proliferate in the host and cause disease. The production of siderophores and outer membrane protein receptors for these molecules is one such adaptation used by various bacterial species.

Alternative mechanisms of iron acquisition exist for certain bacteria, such as *Neisseria meningitidis* and *Pasteurella multocida*, whereby direct contact of transferrin and lactoferrin with surface receptors on the bacterial cell facilitate iron uptake (Tsai et al. 1988, Ogunnariwo et al. 1991). Pathogens in other genera such as *Yersinia* spp. and *Vibrio vulnificus*, can obtain iron directly from heme-containing compounds or ferric citrate (Perry and Brubaker, 1979 and Nishina et al. 1992).

The best characterized siderophore mediated high affinity iron transport system in a marine fish pathogen is the anguibactin-mediated plasmid-encoded system of *Vibrio anguillarum* strain 775 (Crosa, 1989). The expression of this system requires approximately 25 kilobase pairs (kbp) of the 65-kbp plasmid pJM1. Curing *V. anguillarum* strain 775 of the plasmid results in loss of virulence (Crosa et al. 1980), and it has been shown that the portion of the plasmid that plays the major role in the virulence phenotype is the iron uptake region (Actis et al. 1988).

Photobacterium damsela subsp. *piscicida*, which causes a fatal septicemia in a variety of marine fish species, is an emerging problem in the hybrid striped bass mariculture

industry in the state of Louisiana. Previous studies have shown that the bacterium produces a siderophore of unknown class which is believed to contribute to increased virulence of the organism in fish (Magarinos et al. 1994). In order to assess the impact of the siderophore of *P. damsela* subsp. *piscicida* on the virulence of the organism in the hybrid striped bass (*Morone saxatilis* x *Morone chrysops*), a strain with a stable mutation in a siderophore biosynthetic gene was generated. The strain was compared to the wild type strain (LA91-197) in infection trials in juvenile hybrid striped bass to assess changes in virulence and pathogenicity. The mutant was also compared in various tests to assess the changes in biochemical phenotype, enzyme activities and the ability to grow in the presence of the iron chelator EDDA.

Materials and methods

Bacterial strains

A virulent strain of *P. damsela* subsp. *piscicida* (LA 91-197) was removed from LAADL stocks in the ultra-low freezer (-70°C), inoculated into 5 ml of Brain Heart Infusion Broth (BHIB) with 2% NaCl in a 17 X 100 mm test tube and incubated for 24 hours at 25°C. Virulence was confirmed by injecting juvenile hybrid striped bass with 0.1 ml of the culture and re-isolating the bacterium from moribund fish on BHI agar (BHIA) + 2% NaCl.

Detection of siderophores

Siderophore production was detected using a universal chemical assay, chrome azurol-S (CAS) agar of Schwyn and Neilands (1987) as modified by Kokubo et al. (1990). The preparation of CAS agar is described in the following sections.

Basal medium

The basal medium, Minimum Essential Medium - Eagle (MEM) without L-glutamine and containing no phosphates or indicators (Sigma, Catalog No. M-3024) was buffered with 0.1 M PIPES (1, 4 - piperazinediethanesulfonic acid). MEM-PIPES was adjusted to pH 6.8 with sodium hydroxide (NaOH) pellets. Phosphates, used to buffer other minimal media such as M9 (Sambrook et al. 1989), chelate iron and interfere with the detection of siderophores. The medium was deferrated by the addition of 3%, 8-hydroxyquinoline (Waring and Werkman, 1942). The 8-hydroxyquinoline and bound iron were removed by chloroform extractions in a 500 ml glass separatory funnel (Kimax) and the resulting deferrated MEM-PIPES was vacuum filter sterilized into 500 ml plastic containers (Corning Inc., Corning, New York) and stored at 4°C until used to make CAS agar.

CAS Shuttle Solution

CAS Shuttle Solution, used for the detection of siderophores in supernatants and in agar media, was prepared according to the methods of Schwyn and Neilands

(1987). A 6 ml volume of 10 mM hexadecyltrimethylammonium bromide (HDTMA) solution was placed in a 100 ml volumetric flask and diluted with water. A mixture of 1.5 ml iron III solution (1 mM $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 10 mM HCL) and 7.5 ml 2 mM aqueous chrome azurol-S solution was slowly added under stirring. A 4.307 g quantity of anhydrous piperazine was dissolved in distilled water and 6.25 ml of 12 M HCL was slowly added. This buffer solution (pH 5.6) was rinsed into the volumetric flask. CAS Shuttle Solution was completed by adding 5-sulfosalicylic acid to the above solution at a final concentration of 4 mM which was brought to a total volume of 100 ml with distilled water. The solution was stored in a plastic container, in the dark at room temperature until use since the dye solution is light sensitive and temperatures below 25°C may cause crystallization (Schwyn and Neilands 1987). Plastic containers were used in all cases to avoid trace metals that might be encountered in glassware.

CAS agar

CAS agar was produced by heating MEM-PIPES for 30 minutes in a water bath at 50°C to drive off residual chloroform from the iron extractions. Six grams of Bacto agar (Difco) was dissolved in 100 ml of MEM-PIPES by heating on a hot plate for 10 minutes to dissolve the agar and then autoclaved for 15 minutes at 121°C and 15 psi. The melted agar was slowly added to 350 ml of MEM-PIPES

preheated to 50°C and the medium held in a 500 ml plastic container in a 50 °C water bath. Shuttle Solution (50 ml) was slowly added to the medium to avoid foaming and poured into plastic petri plates (20 ml per plate). For selection of transformants and transposition, kanamycin was added to the medium to achieve a final concentration of 50 µg/ml, 80 µg/ml or 100 µg/ml prior to pouring plates. Plates are light blue in color when cool and isolated colonies producing siderophore are surrounded by an orange-yellow halo.

Bacterial transformation and mutagenesis

The method of bacterial transformation used in this study was electroporation and the method of gene inactivation was transpositional mutagenesis. The introduction of the transposon into recipient cells was accomplished by delivery on suicide plasmid vectors.

Competent cells

Standard procedures as outlined for Gram negative bacteria in the Gene Pulser® pulse controller instruction manual (Bio-Rad Laboratories, Richmond, California) and for *Escherichia coli* by Dower et al. (1988) were used in an attempt to make competent cells. When these methods were unsuccessful, an alternative method was used. Briefly, a 5 ml broth culture of MEM-PIPES was inoculated with a single colony of strain 91-197, previously determined to be virulent and siderophore positive, and grown for 24 hours

at 25°C. One ml of this culture was inoculated into 50 ml BHI + 2% NaCl broth and cultured at 27°C for 4 hours. The broth culture was centrifuged at 4000 x g for 15 minutes at 4°C in a Sorvall RT6000 refrigerated centrifuge (DuPont Co., Biomedical Division, Newtown, Connecticut) and the cells resuspended and washed twice in 2 mM Hepes buffer + 100 mM sucrose at pH 6.8. Cells were resuspended a final time in 3 ml of the above solution, distributed into eppendorf tubes at 500 µl per tube, and stored at -70°C in an ultra-low freezer until needed.

Electroporation

Electroporation was carried out in a Gene Pulser[®] apparatus (Bio-Rad) coupled with a pulse controller unit (BioRad) in 0.2 cm cuvettes using the following settings; gene pulser (2.0 KV), pulse controller (200 ohm) and capacitance (25 microfarads). The time constant and actual voltage and capacitance were measured following each electrical discharge. The modified transformation buffer described above provided osmotic stability for the cells and had limited electrical conductivity which prevented arcing.

Minitransposons and delivery vehicles

The plasmid pNK2859 is a pBR322 derivative that carries a marker for ampicillin resistance and serves as the delivery vehicle for a 1.8 kb mini-transposon (Tn10 derivative, 103) carrying the gene for kanamycin resistance

originally excised from Tn903 (Kleckner et al. 1991). The mini-transposon is bounded by identical inverted repeats of the outermost 70 bp of IS10R and has the transposase genes *ats1* *ats2* located in cis under control of Ptac, a strong IPTG inducible promoter. The transposase genes have been mutated to produce a transposase that alters the target specificity (ATS) of the IS elements resulting in a more random distribution of insertion sites in the chromosome with little decrease in transposition activity. The Kan^R fragment is oriented in the transposon backbone so that the Kan^R gene is transcribed in the same direction as the Ptac promoter. The plasmid vehicle becomes linearized when the transposase excises the transposon and is degraded by nucleases of the recipient cell. The plasmid pNK2859 is grown in *Escherichia coli* strain DH5- α .

Plasmid pEIS, constructed in the laboratory of Dr. Richard Cooper, is a hybrid plasmid between pNK2859 and pGP704 and contains the same minitransposon cassette as pNK2859 (Cooper personal communication, 1995). The suicide plasmid pGP704 (Miller and Meckalanos, 1988), contains the R6K origin of replication, and requires the R6K specific replication protein π produced by the *pir* gene in phage λ to replicate in a host cell. Plasmid pGP704 cannot be maintained except in certain lambda lysogenic strains of *E. coli* that produce the π protein. Plasmid pEIS was constructed by cutting the transposon cassette out of

pNK2859 with the restriction enzyme EcoR1 and ligating the 4.2 kb fragment into the muticloning site in pGP704. This results in a transposon inducible by IPTG, carried on a 7.9 kb suicide plasmid vehicle that cannot replicate in a host bacterium that does not produce the π protein. The plasmid pEIS is maintained in *E. coli* strain SY327 (λ pir), a lambda lysogen.

Transformation and mutagenesis protocol

A tube containing concentrated competent cells was removed from the ultrafreezer, thawed at room temperature and placed on ice. Forty μ l of cells were transferred to an eppendorf tube on ice and 1 μ l of plasmid DNA solution pNK2859 (1.7 μ g/ μ l) or 3 μ l pEIS (.37 μ g/ μ l) was added to a final concentration of 0.04 μ g/ μ l pNK2859 or 0.02 μ g/ μ l pEIS. The cell suspension/DNA mixture was placed between the electrodes in the electroporation cuvette and a pulse applied. Immediately following the pulse, 1 ml of BHI broth or MEM-PIPES and 10 μ l of IPTG were added to the the cuvette and the mixture transferred to a 17 x 100 mm glass test tube. The mixture was incubated in a roller apparatus (Cel-Gro, Lab Line Products, Melrose Park, Illinois) at 30°C for 2 hours. Ten-fold dilutions of the mixture were made in MEM-PIPES to determine the dilutions that would give countable colony forming units (CFU) on CAS agar plates and CAS/KAN plates. Dilutions were plated on non-selective CAS agar to determine total cell numbers.

Transformation efficiency was calculated as CFU/ μ g of plasmid DNA added. Transformation frequency was calculated as transformants/survivors (total cells counted on plates). Colonies that grew on CAS/KAN agar plates were screened following 72 hours of incubation at 27°C for colonies that failed to produce a yellow-orange halo, indicative of a siderophore negative phenotype.

Selection and evaluation of mutant

A mutant generated using the above procedures, designated LSU-P1, was evaluated to insure that the siderophore negative phenotype was the result of transposon insertion and not the result of a spontaneous mutation in siderophore production in combination with a spontaneous mutation for Kan^R or failure of the delivery vehicle to suicide. Plasmid minipreps of the mutant produced by the above procedure were loaded into 0.7% agarose gels along with purified pNK2859 DNA or pEIS DNA to insure that the delivery plasmid was not maintained. In addition the mutant was plated on LB medium (Sambrook et al. 1989) supplemented with 2% NaCl and 200 μ g/ml ampicillin to establish ampicillin sensitivity. Ampicillin resistance indicated maintenance of the shuttle vector because pNK2859 contains a marker for Amp^R outside of the transposon cassette.

Mutant LSU-P1, wild type, and spontaneous Kan^R strains were also tested for insertion of the transposon and

maintenance of the Kan^R marker by Polymerase Chain Reaction (PCR) analysis. Minipreps of strains prepared with the Qiagen system (Qiagen Inc., Chatsworth, CA.) containing both genomic and plasmid DNA, were mixed with primers KAN-1 and KAN-2 in a reaction mixture and loaded into a programmable thermocycler (Perkin Elmer Inc., Roche Molecular Systems, Branchburg, New Jersey) under optimized conditions. The primers, synthesized by GeneLab, Department of Veterinary Microbiology and Parasitology, Louisiana State University, Baton Rouge, Louisiana, consisted of the following 5' TO 3' sequences. KAN-1: CAA CAA AGC CAC GTT GTG TCT CAA AAT CTC. KAN-2: TCA AGT CAG CGT AAT GCT CTG CCA GTG. PCR reactions contained 1 μ l template (.0675 μ g/ μ l), 10 μ l 10X Vent buffer, 1.5 μ l Vent polymerase, 3 μ l KAN-1 (10 μ M), 3 μ l KAN-2 (10 μ M), 8 μ l dNTP mix (each dNTP = 1 mM), 0.5 μ l MgSO₄ (100mM), 2 μ l DMSO and 71 μ l dH₂O and were overlaid with 70 μ l sterile mineral oil. The thermocycler was programmed for a hot start at 95°C followed by 95°C for 30 seconds, 64°C for 30 seconds and 72°C for 1 minute. The cycle was repeated for 30 cycles and was then held at 4°C.

Minimal inhibitory concentration (MIC) of EDDA

Strains producing siderophores can grow in the presence of high concentrations of EDDA (Biosca and Amaro 1991). Kanamycin resistant and siderophore negative strains produced from wild type strain 91-197 using pNK2859

(M1001) or pEIS (LSU-P1) were tested to determine the minimal inhibitory concentration of the iron chelator ethylene-diamine-di-(O -hydroxyphenylacetic acid) (EDDA) in 24 well plates. Concentrations of EDDA in 1 ml MEM-PIPES per well, ranged from 13 μ M to 14 mM.

Virulence and pathogenicity trials

Immersion

The virulence of mutant LSU-P1 was assessed in the immersion infection model developed for hybrid striped bass (Chapter 3). Juvenile hybrid striped bass ranging in size from 20 to 31 grams were stocked at 10 fish per 70 liter system and challenged by immersion at levels ranging from 8.0×10^6 CFU/ml to 1000 CFU/ml to determine the LD₅₀. When no mortality occurred in the LD₅₀ trial, three systems containing ten fish each, were exposed to 6.0×10^7 CFU/ml by immersion and were sampled at 3, 6 and 12 hrs post infection (PI) and every 24 hours thereafter. At each sample period three fish were removed from the tanks, anesthetized with MS-222 and necropsied. Gill tissues obtained at each sample period during the first 48 hours were fixed in gluteraldehyde for EM processing.

Injection

Ten fish ranging in weight from 20 to 35 grams were infected by intraperitoneal (IP) injection with 2.0×10^9 CFU/fish (0.1 ml/fish) of mutant LSU-P1. Twenty-five juvenile hybrid striped bass ranging in weight from 20 to

43 grams were infected by IP injection in a second trial with 2.0×10^8 CFU/fish (0.1 ml/fish) of either mutant LSU-P1 or wild type 91-197 and stocked into two different 500 liter recirculating systems. Three fish were sampled from each system at 24 hour intervals, anesthetized in MS-222, and tissues aseptically removed for bacterial enumeration.

Resistance to bactericidal effect of normal HSB serum

Mutant LSU-P1 was compared to the wild type strain 91-197 and a complement sensitive strain, *Escherichia coli* strain QC-779, in a bactericidal assay. Blood serum was collected from 6 fish ranging in weight from 200 - 250 grams in plain glass 10 ml vacutainers (Becton Dickenson). The blood was allowed to clot at room temperature for 1 hour and was refrigerated over night at 4°C. The serum was collected the next day, pooled, and stored at 4°C. Prior to performing the assay, a 1 ml aliquot of pooled serum was heat inactivated for 30 minutes at 56°C. The assay was performed in a 24 well tissue culture plate (Corning) by the addition of suspensions of each bacterial strain ($\sim 2.0 \times 10^8$ cfu/ml) in saline to three different wells (100 μ l/well). One hundred microliters of normal serum was added to each well of a dilution series of each bacterial suspension made in sterile saline through 10^{-2} . Three sets of dilutions were made in a similar fashion with the same three bacterial strains with 100 μ l heat inactivated serum added to each well in the series. Samples were taken from

the wells at 30 minutes, 1 hour, 2 hours and 4 hours following addition of the serum for bacterial enumeration.

Results

Transformation using pNK2859

The plasmid pNK2859 was successfully electroporated into competent *P. damsela* subsp. *piscicida* in five initial experiments. Electroporated cells incubated in 1 ml of BHI broth in the presence of IPTG with or without DNA produced counts of $\sim 5.0 \times 10^7$ on BHI agar and CAS agar with no Kan. On plates with Kan selection, cells electroporated with pNK2859 produced countable colonies at the 10^{-4} dilution, representing 2.8×10^6 Kan^R cells/ml in the recovery broth. Of approximately 4100 Kan^R colonies screened on CAS agar, only one was found to be siderophore negative. No spontaneous siderophore negative colonies were found when over 10,000 competent cells were screened on non-selective CAS agar. Unfortunately, mutants produced with pNK2859 were demonstrated to maintain the plasmid at a low copy number and the growth was only slightly inhibited on plates containing 200 μ g ampicillin. Thus, it was not possible to confirm that transposition had occurred and the genotype of the mutants remained in question. This is unfortunate because pNK2859 transformed *P. damsela* subsp. *piscicida* with an efficiency of $7.09 \times$

10^7 transformants per μg DNA and a transformation frequency of 5.5%.

Transformation and mutagenesis using pEIS

The plasmid pEIS was successfully electroporated into competent *P. damsela* subsp. *piscicida* in two preliminary experiments, but the transformation efficiency was very low. Production of new competent cells of a higher cell density and harvested from culture while the cells were still in log phase improved the efficiency slightly. Electroporated cells recovered in 1 ml of MEM-PIPES in the presence of IPTG with or without DNA produced counts of approximately 6.0×10^7 cfu/ml on BHI + 2% NaCl and CAS plates with no antibiotic selection. Countable colonies occurred on BHI/KAN selection plates when 100 μl of undiluted cell suspension (approximately 6×10^6 cells) was spread per plate. The average number of mutants generated from plating 100 μl of an undiluted suspension of electroporated cells was 15 per plate. Transposition efficiency was very low and was calculated to be $1.3 \times 10^2/\mu\text{g}$ DNA. Kanamycin resistant colonies were patched to CAS/KAN plates and one siderophore negative mutant was detected. The mutant, designated LSU-P1, grew in the presence of 80 - 100 $\mu\text{g/ml}$ kanamycin, but failed to grow on BHI + 2% NaCl + 200 $\mu\text{g/ml}$ ampicillin.

Evaluation of siderophore negative mutant LSU-P1

A plasmid miniprep of LSU-P1 failed to show any bands corresponding to pEIS or any other unusual bands. Strain LSU-P1 failed to grow in the presence of 200 $\mu\text{g/ml}$ ampicillin and had an MIC calculated to be 0.4 $\mu\text{g/ml}$, which is similar to 0.2 $\mu\text{g/ml}$ for the wild type. PCR analysis was conducted using DNA from strain LSU-P1, wild type *P. damsela* subsp. *piscicida* 91-197 DNA, and DNA from a spontaneous Kan^R strain isolated during one of the electroporation trials. Purified pEIS plasmid DNA was included as a positive control. Only the purified pEIS DNA and DNA extracted from LSU-P1 were positive for primer annealing and generation of the predicted 1.0 kb PCR product in the reaction (Figure 1). This confirmed the insertion of the transposon cassette carrying the *kan* gene in the genome of LSU-P1.

The minimal inhibitory concentration of EDDA for wild type 91-197, LSU-P1 and M1001 was determined to be 7.1 mM, 221 μM , and 887 μM respectively. This corresponds to a 32-fold difference between the wild type and LSU-P1. Strains not synthesizing siderophore cannot obtain iron bound to EDDA as efficiently as siderophore positive strains.

Virulence and pathogenicity of LSU-P1

Siderophore negative mutant LSU-P1 was found to be non-virulent for hybrid striped bass by either immersion or injection routes of infection. Following immersion

exposure, no bacteria were isolated from the blood, spleen, liver or kidney for up to one week PI.

Two separate closed recirculating systems stocked with 25 fish each and challenged with 2.0×10^8 CFU/fish of either 91-197 or LSU-P1 gave dramatically different results. Fish injected with the wild type strain were all dead by 30 hours PI and bacteria were isolated from the liver, kidney, spleen and blood in high numbers. Fish injected with the mutant did not show clinical signs and, although most fish cultured positive for the organism in subsequent daily samples, only one fish showed abnormal pigmentation and high recovery of bacteria upon necropsy and no other fish became sick or died during the trial, which was terminated 2 weeks PI. No bacteria were detected from the tissues of any fish after the sixth day PI.

Gill tissues sampled at 3, 6, 12, 24 and 48 hours post-immersion infection were found to contain evidence of bacterial penetration of the epithelium but no bacterial colonies or associated tissue damage developed.

Resistance to the bactericidal effect of normal HSB serum

Mutant LSU-P1 and wild type strain 91-197 were both found to resist the bactericidal effects of normal hybrid striped bass serum. Complement sensitive control strain *E. coli* QC 779 was killed within the first 30 minutes of the test in the normal serum but was not killed in the heat inactivated serum.

Discussion

The development of a procedure for making competent cells was an initial obstacle which had to overcome before transformation of *P. damsela* subsp. *piscicida* by electroporation was possible. Early attempts at washing and suspending cells in traditional transformation buffers and then in distilled water and 10% glycerol failed because *P. damsela* subsp. *piscicida* is a halophilic organism and the osmotic stresses limited its survival in distilled water. Cell lysis caused cell viability to be extremely low in initial preparations. The use of transformation buffer containing 2 mM Hepes and 100 mM sucrose and 10% glycerol allowed multiple vials of high viability cells to be frozen back and used as needed. In addition, the ability of the cells to be transformed was greatly influenced by age of the culture. The change in morphology from rods in early log phase culture to coccoid forms in older cultures is a characteristic of *P. damsela* subsp. *piscicida* noted by several authors (Gauthier et al. 1995; Simidu and Egusa, 1972). The young, longer rods that predominate in cultures after 4 to 5 hours of incubation are apparently much more suitable for transformation.

Although transformation and mutation of *P. damsela* subsp. *piscicida* by electroporation with pEIS proved to be of low efficiency, one stable siderophore negative mutant was produced. The failure of mutant LSU-P1 to establish

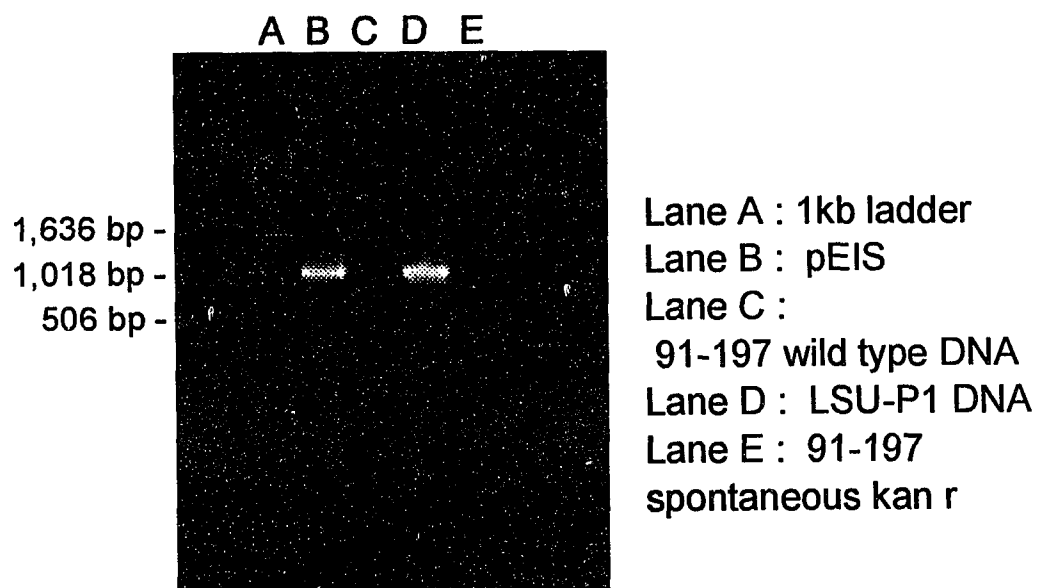


Figure 15. Agarose gel electrophoresis of PCR products generated from mutant (LSU-P1) and wild type (91-197) strains of *Photobacterium damsela* subsp. *piscicida* with a positive control

infection in hybrid striped bass by immersion indicates the siderophore may be important in invasion or early establishment of infection. The ability of the mutant to persist for up to 6 days PI by IP injection may be the result of resistance to the bactericidal effects of serum and, possibly, an intracellular mode of survival within macrophages. Without the ability to proliferate in the blood the organism would be gradually cleared from the fish.

The potential usefulness of LSU-P1 as a live attenuated vaccine might be limited because the most economical method of exposure, immersion, is ineffective in establishing a systemic infection. The mutant might persist long enough following injection to elicit protective immunity, however, this concept has not been tested. Injection of a killed vaccine has been determined by some producers to be a viable economic alternative to conventional vaccination by dips or baths (Fernandez 1995, personal communication). This is an area for future research with siderophore mutants.

SUMMARY

Thirty-two cultures of the marine bacterium *Photobacterium damsela* subsp. *piscicida* isolated from disease outbreaks in populations in hybrid striped bass cultured on Louisiana mariculture farms over the period from December, 1990 to December, 1995 by the Louisiana Aquatic Animal Diagnostic Laboratory were compared to each other and strains from Japan, Greece, Israel, and Chesapeake Bay, USA, using a variety of molecular, biochemical, antimicrobial, and ELISA techniques.

The Louisiana isolates are identical to each other and with strains from other geographic areas in the areas of biochemical phenotype and enzymic activity. The only areas where variability exists is in the native plasmid profile and in antimicrobial susceptibility. The presence of two large plasmid bands of approximately 30 and 40 kb and two smaller plasmid bands corresponding to 8 and 5 kb are unique to Louisiana strains. Differences in antimicrobial susceptibility were seen among the strains tested.

Infectivity trials were carried out to determine the immersion and oral LD₅₀ for *P. damsela* subsp. *piscicida* in juvenile hybrid striped bass. Experimental infection could not be achieved by oral administration but the LD₅₀ by immersion is less than 1250 CFU/ml of tank water.

To investigate the progress of infection following immersion infection, two trials utilizing a low dose (6000

CFU/ml) and a high dose (12000 CFU/ml) were conducted. The sequential bacteriology and histopathology of the disease was determined by sacrificing fish at daily (24 hour) intervals and enumerating bacteria in samples of liver, kidney, spleen and blood. Remaining portions of these organs as well other tissues from the fish were collected, fixed, processed, and sectioned for light microscopy.

Sequential bacteriology revealed that at the high dose the bacterium was found in the spleen and gills as early as 24 hours post infection (PI) and in the kidney and blood by 48 hours PI. The liver was the last organ to be colonized, culturing positive at 72 hours PI. Following low dose immersion infection the same pattern emerged but bacteria were not found in any of the organs until 72 hours PI. The spleen contained a significantly higher number of cells than any other organ at all sampling time periods. Histological examination of the gills using semi-thin sections showed high numbers of bacterial cells invading and colonizing the gill tissue at 24 hours PI. Death of the fish appears to result from necrosis of the gill lamellae and the blockage of blood flow to the gills by masses of bacteria in the capillaries and sinusoids.

Pathogenic isolates of *P. damsela* subsp. *piscicida* were shown to produce an iron sequestering system (siderophore) that is believed to function as a virulence factor in infections of hybrid striped bass. A

minitransposon was used to mutagenize wild type strain 91-197 of *P. damsela* subsp. *piscicida* by introduction on pEIS, a suicide delivery plasmid following electroporation. A stable mutant strain (LSU-P1) deficient in siderophore biosynthesis was generated using this system. LSU-P1 was evaluated in the immersion challenge model developed in Chapter III and found to be non-virulent in juvenile hybrid striped bass. The mutant was found to be phenotypically identical with the wild type strain 91-197 in biochemical, enzymic and plasmid profile. LSU-P1 differed from the wild type in being Kan^R (a result of the transposon insertion) and 32 times more sensitive to the iron chelating agent EDDA. The insertion of the transposon was confirmed by PCR analysis using two different primers to the *kan* gene located in the transposon.

Future research should focus on several avenues opened by this study. The immersion challenge system provides a useful model to evaluate the effects of vaccination and drug administration in hybrid striped bass as well as evaluating other mutants that will be produced. The system developed for transpositional mutagenesis should result in generation of other siderophore mutants that will help in mapping and characterizing the gene(s) encoding the molecule and it's outer membrane protein receptors in *P. damsela* subsp. *piscicida* and also in the generation of other virulence factor mutants.

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VITA

John P. Hawke was born to Evelyn and Jack Hawke on November 5, 1949 in Birmingham, Alabama. John graduated from Ensley High School in Birmingham in 1967. Following high school he enrolled in Jefferson State Junior College in Pinson, Alabama where he received the Associate in Science degree in Biological Sciences in 1970. John entered Auburn University in the fall of 1970 and began studies in the Department of Fisheries and Allied Aquacultures. He received his Bachelor of Science degree in Fisheries Management in 1972 and his Master of Science degree in 1974 in Fisheries Biology.

John worked as a fish disease diagnostician with the Southeastern Cooperative Fish Disease Project at Auburn University until 1989 when he accepted a position as Marine Biologist II with the Alabama Department of Conservation, Marine Resources Division.

In 1986 John accepted a position as Research Associate with the Department of Veterinary Microbiology and Parasitology, Louisiana State University and attained the rank of Instructor in 1990. In 1986 he began working toward the Doctor of Philosophy degree in the Department of Veterinary Microbiology and Parasitology. He is married to Rebecca (Bullock) Hawke and has two daughters, Autumn, age 21 years, Rachel, age 19 years, and a son, Phillip, age 12 years.

DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate: John P. Hawke

Major Field: Veterinary Medical Sciences

Title of Dissertation: Importance of a Siderophore in the Pathogenesis and Virulence of Photobacterium damsela subsp. piscicida in Hybrid Striped Bass (Morone saxatilis X Morone chrysops)

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March 29, 1996
